



Microbial water quality within Recirculating Aquaculture Systems

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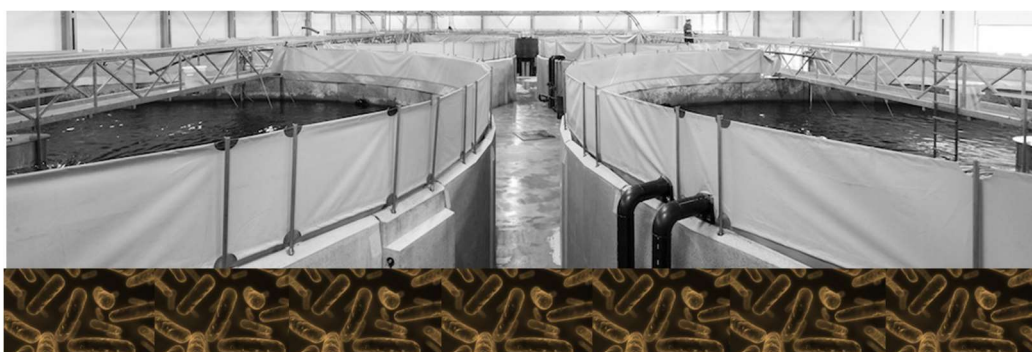
Microbial water quality within recirculating aquaculture systems

PhD Thesis



Written by Paula A. Rojas-Tirado
Defended 1 March 2018

Microbial water quality within recirculating aquaculture systems



Paula A. Rojas-Tirado

PhD Thesis
November 2017

Technical University of Denmark
National Institute of Aquatic Resources
Section for Aquaculture

Preface

This PhD thesis is submitted as a partial fulfilment to attain the Doctor of Philosophy degree (Ph.D). This thesis presents the research carried out during my enrolment as a PhD student at the Section for Aquaculture, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark in Hirtshals, Denmark.

The main supervisor of this PhD study was Senior Scientist, Dr. Lars-Flemming and the co-supervisor was Per Bovbjerg Pedersen, Head of Section.

The thesis is based on the topic of microbial water quality assessment in freshwater recirculating aquaculture systems and includes three scientific manuscripts:

- Paper I.** Rojas-Tirado, P., Pedersen, P.B., Pedersen, L-F. 2017. Bacterial activity dynamics in the water phase during start-up of recirculating aquaculture systems. *Aquacultural Engineering* 78: 24-31.
- Paper II.** Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L-F. 2018. Changes in RAS microbial water quality associated with changes in feed loading. Submitted to *Aquacultural Engineering*.
- Paper III.** Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L-F. 2018. Monitoring abrupt changes in bacteria within biological stable RAS water. Manuscript.

During my PhD, the research results have presented at national and international conferences.

Conferences:

Rojas-Tirado, P., Pedersen, P.B., Pedersen, L-F. 2015. Microbial water quality dynamics in RAS during system start-up. 3rd NordicRAS 2015, Molde – Norway. Oral presentation + Book of Abstracts.

[Online Abstract]:

3rd NordicRAS Workshop on Recirculating Aquaculture Systems

Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L-F. 2016. Effect of feed loading on microbial water quality in RAS. The 11th International Conference on Recirculating Aquaculture (ICRA), Roanoke, USA. Oral presentation + Book of Abstracts.

[Online Abstract]:

The 11th International Conference on Recirculating Aquaculture (ICRA)

Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L-F. 2017. Monitoring abrupt changes in bacteria within biological stable RAS water. 4th NordicRAS 2017, Aalborg – Denmark. Oral presentation + Book of Abstracts.

[Online Abstract]:

4th NordicRAS Workshop on Recirculating Aquaculture Systems

The work has also included additional collaboration

Work collaboration:

Becke, C., Schumann, M., Steinhagen, D., Rojas-Tirado, P., Geist, J., Brinker, A. 2017. Combined effects of chronic exposure to suspended solid load and increased unionized ammonia concentration on the physiology and growth performance of rainbow trout (*Oncorhynchus mykiss*). Article in prep.

Spiliotopoulou, A., Rojas-Tirado, P., Chhetri, R.K., Kaarsholm, K.M.S., Martin, R., Pedersen, P.B., Pedersen, L-F., Andersen, H.R. 2017. Optimum ozonation of freshwater pilot recirculating aquaculture system – Water quality. Article Submitted to Water Research Journal.

Both work collaborations were presented at the 4th NordicRAS workshop 2017.

[Online Abstract]: 4th NordicRAS Workshop on Recirculating Aquaculture Systems

November 2017

Paula A. Rojas-Tirado

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Special thanks to Ulla Sproegel, Brian Møller, Dorte Frandsen, Ole Madvig Larsen, Rasmus Frydenlund Jensen and the technicians that are not part of the team anymore. I appreciate all your effort and kindness. Your contribution is a great part of this PhD study.

I would like to extend my gratitude to Professor Olav Vadstein from NTNU, for inspiring me with his knowledge in relation to the microbials world in aquatic systems and for his valuable contribution to my work.

I would like to thank all of my colleagues at DTU Aqua Hirtshals for helping me in different tasks. Particular thanks to my colleagues Carlos Letelier and Javed Kahn for revising this thesis. Thank so much for your valuable input, comments and suggestions guys. To my friends in Hirtshals, you all turn Hirtshals into a great place. To my friends in Chile and Norway, thank you for your calls, messages and laugh.

I owe a big thank to Mireya Gordo who has been a great support during this phase of my life.

Finally, I would like to thank my mother for all her love and support during my whole life but also during this adventure. To my father, who taught me that great things are achieved with effort and perseverance. To my brothers and nephew whom I love.

To all of you,

Thank you.

*Paula Andrea Rojas Tirado
Hirtshals, 30 November 2017.*



English Abstract

Empirical observations suggest causality between water quality and fish performance. Direct monitoring of microbial water quality (MWQ) is presently not part of recirculating aquaculture systems (RAS) management. Current standard methods to assess microbial water quality in RAS under operational conditions are based on direct predictive tools such as turbidity or visual observation on fish performance. Factors affecting MWQ in RAS include but are not limited to organic matter input and accumulation, environmental conditions, biological processes, RAS treatment units and management.

As RAS development takes place at a high pace, there is an urgent need to understand the link between operational factors and microbial dynamic. In order to achieve this, rapid and reliable monitoring tools are required.

The aim of this PhD thesis was to assess selected aspects of microbial water quality submitted in freshwater RAS experimentally controlled under different conditions. Microbial water quality changes were measured through a set of new, rapid, culture-independent and reliable methods such as Bactiquant®, hydrogen peroxide (HP) degradation assay and flow cytometry.

The present thesis encloses three scientific articles and unpublished data collected during the last three years. The three articles are related to: 1) bacterial activity dynamic in the water phase during start-up of RAS; 2) detection of changes in RAS MWQ associated to changes in feed loading; 3) monitoring of abrupt changes in MWQ within RAS water.

The first manuscript (Paper I) evaluated bacterial activity development in six identical , pilot scale freshwater RAS stocked with rainbow trout (*Oncorhynchus mykiss*) during a three months period from start-up. The systems were operated under constant conditions in terms of feed and water exchange. Bacterial activity was assessed with a new method called Bactiquant®, which measures bacterial activity indirectly by targeting a specific enzyme from the hydrolase class. The results showed that during start-up, bacterial activity increased (quantified by Bactiquant®) with substantial fluctuations (variation) between RAS. After a three weeks period, the bacterial activity stabilized, which was correlated to both particulate and dissolved fractions of organic matter measured as chemical oxygen demand (COD).

The second study (Paper II) investigated changes in RAS microbial water quality associated with changes in the feed loading. After an experimental stabilization period, under constant conditions, the RAS were divided into three treatment groups according to the feed loading. The effect of feed loading was evaluated based on duplicate RAS: i) unchanged (3.13 kg/m³); ii) stopped feeding (0 kg/m³); and iii) doubled feeding (6.25 kg/m³). Microbial water quality was assessed in terms of bacterial activity with Bactiquant® and HP degradation assay and bacterial abundance with flow cytometry. The overall results showed that microbial water quality responded (directly) positively to feed loading changes. Bacterial activity was highly related to the accumulation of particulate organic matter whereas abundance of free-living bacteria was associated with the dissolved organic matter content. A delay in bacterial activity and abundance response in the water phase was observed and it was suggested that the biofilter buffered transient and prolonged changes by biofilm/water phase interactions.

The third study (Paper III) was performed in an extensive experimental setup including twelve separate and identical freshwater RAS. The 1.7 m³ pilot scale RAS with rainbow trout were kept under constant conditions and managed after predefined protocols for five months. The aim of this study was to evaluate the effects of different levels of easy biodegradable substance on MQW in RAS subjected to acetate spiking trials. For three consecutive days, acetate was spiked (daily pulse addition of 40 mg/l acetate) into the fish tanks and associated bacterial activity was investigated before, during and after addition in both batch and full-scale experiments. Microbial water quality was assessed in terms of activity (Bactiquant® and HP degradation assay) and abundance (flow cytometry). The bacterial dynamics and potential influence by the biofilter, was also evaluated within the full scale spike experiments. The experimental setup group consisted of four treatments: i) control RAS with biofilter, ii) control RAS without biofilter, iii) RAS with biofilter, spiked with acetate; and iv) RAS without biofilter, spiked with acetate. Each treatment was evaluated in triplicated RAS. The main results showed that bacteria were substrate limited but as soon as the systems were spiked with the readily, easy degradable carbon source, bacterial increased in terms of activity and number. The biofilter related communities were found to be the main source of consumption of the additional carbon source, suppressing the growth of bacteria in the water column.

In conclusion, both of the rapid and simple methods - Bactiquant® and HP degradation assay - proved to provide a reliable, broad picture regarding microbial activity, by taking into consideration both free-living bacteria and particles-associated bacteria. Bacterial activity was related to the presence of particulate organic matter. Flow cytometry quantified the numbers of free-living cells in the water phase, which was highly associated with the dissolved fraction of the readily available organic matter.

Furthermore, this PhD study elucidated that when monitoring bacteria in the water phase there are two main interactions that have to be taken into consideration in future studies: i) biofilter bacterial population vs suspended bacterial population (both free-living and particle-associated bacteria); and ii) free-living bacterial population vs particle associated bacterial population.

Dansk Resumé

Overvågning af mikrobiel vandkvalitet (MV) i recirkulerede anlæg indgår endnu ikke som en naturlig del af den daglige driftsmonitoring. Eksisterende metoder til vurdering af mikrobiel vandkvalitet i recirkulerede akvakultur systemer (RAS) er baseret på indirekte målinger som eksempelvis vandets turbiditet eller observationer af fiskenes ædelyst og adfærd. Der er en lang række af faktorer der påvirker den mikrobielle vandkvalitet i RAS, herunder input og akkumulering af organisk materiale (foder og fækalier), miljøbetingelser, samt mekaniske og biologiske processer i RAS-enheder og drift heraf.

Recirkulerende akvakultur systemer udvikles i høj fart og derfor er det et presserende behov for at forstå sammenhænge mellem driftsfaktorer og den mikrobiel dynamik i anlægsvandet. For at kunne opnå dette, kræves der overvågningsværktøjer der på kort tid kan give pålidelige målinger.

Formålet med denne PhD-afhandlingen var at vurdere udvalgte aspekter af mikrobiel vandkvalitet i ferskvands-RAS under forskellige kontrollerede, eksperimentelle betingelser. Ændringer i mikrobiel vandkvalitet blev målt ved brug af nye, metoder som Bactiquant®, hydrogen peroxid (HP) omsætning og flow cytometri.

Afhandling indeholder tre videnskabelige artikler og upublicerede data baseret på tre års arbejde. De tre artikler omhandler:

- 1) undersøgelser af dynamikken af bakteriel aktivitet i vandfasen under opstart af RAS;
- 2) undersøgelser af MV i RAS MV ved forskellige foder belastning;
- 3) Undersøgelse af effekter af substrat tilførsel på den mikrobielle vandkvalitet i RAS;

Den første undersøgelse (Paper I) evaluerede udviklingen af bakteriel aktivitet i seks identiske, pilotskala ferskvands RAS med regnbueørred (*Oncorhynchus mykiss*) over tre måneders periode fra opstart. Anlæggene blev holdt under konstante betingelser i forhold til foder og vandskifte. Vandfasens bakteriel aktivitet blev målt med en ny metode kaldet Bactiquant®, som måler summen af bakteriernes aktivitet indirekte ved hjælp af et specifikt hydrolase enzym. Resultaterne viste, at der i RAS-anlæggenes start fase, var en generel stigende udvikling i bakterieaktiviteten med tydelig en vis variation mellem de enkelte RAS. Efter en periode på tre ugers, stabiliserede den bakterielle aktivitet i vandfasen, og viste sig at være positiv korreleret med både partikulært og opløst organisk materiale målt som kemisk iltforbrug (COD).

Den anden undersøgelse (Paper II), undersøgte forskellige aspekter af den mikrobielle vandkvalitet i RAS-anlæg i forbindelsen med ændringer i foderbelastningen. Efter en eksperimentel stabiliseringsperiode under konstante betingelser, blev anlæggene opdelt i tre behandlingsgrupper. Effekten af 3 forskellige niveauer af foderbelastning blev vurderet i pilotskala RAS (n=6): i) uændret indfodring (3.13 kg/m³); ii) ophørt fodring (0 kg/m³); og iii) fordobling i indfodring (6.25 kg/m³). Mikrobiel vandkvalitet blev undersøgt på baggrund af bakteriel aktivitet målt med Bactiquant® og HP-omsætningsstest og suspenderede celler blev kvantificeret med flow cytometri. De generelle resultater var, at den mikrobielle vandkvalitet ændrede sig og var ligefrem proportionel med foder belastningerne. Den bakterielle aktivitet var stærkt korreleret til partikulært organisk stof, mens suspenderende bakterier var forbundet med indholdet af det opløste organisk materiale.

En tilsyneladende forsinkelse i bakteriel aktivitet og bakterier antallet i vandfasen blev observeret, og forklaret med at biofilteret regulerer ændringer ved interaktioner mellem biofilm og vandfasen.

Den tredje undersøgelse (Paper III) blev udført i et omfattende eksperimentel forsøgsopsætning med tolv separate og identiske 1.7 m³ ferskvands RAS-anlæg. RAS-anlæggene med regnbueørred blev holdt under konstante forhold ifølge foruddefinerede protokoller over en periode på fem måneder. Formålet med dette studium var at undersøge betydningen af at tilføre et nemt biologisk nedbrydeligt substrat over for den mikrobielle vandkvalitet. Dette foregik ved at dagligt at tilsætte daglig acetat (puls spike svarende til 40 mg/L acetat) over tre på hinanden følgende dage.

Effekterne af denne substrat tilsætning på den bakterielle aktivitet blev undersøgt før, under og efter tilsætning i både batch- og fuldskalaforsøg. Mikrobiel vandkvalitet blev vurderet både i form af aktivitet (HP omsætningstest) og ved brug af flow cytometriske metoder til at kvantificere suspenderede bakterier. Den bakterielle dynamik ved substrattilsætning og i forhold til biofilterets betydning blev også evalueret i fuldskala forsøget. De fire eksperimentelle grupper (n=12) bestod af følgende: i) kontrol RAS med biofilter ii) kontrol RAS uden biofilter iii) RAS med biofilter, tilsat acetat; og iv) RAS uden biofilter, tilsat acetat. Hver behandling foregik i RAS i triplikater. Hovedresultaterne viste, at bakterier i RAS vandet var substrat begrænsede, men så snart acetat var tilsat, steg bakterierne i aktivitet og i antal. De biofilterrelaterede mikrobielle samfund viste sig at være den primære kilde til forbrug af den ekstra kulstofkilde, og undertrykkede væksten af bakterier i vandfasen.

Helt overordnet viste undersøgelserne, at både Bactiquant® og HP omsætningstest med fordel kan anvendes til at give et hurtigt og stabilt mål for den mikrobielle aktivitet i vandfasen. Begge assays har den fordel at både frit levende bakterier og partikelassocierede bakterier indgår i aktivitetsbetømmelserne, og det er vigtigt, idet bakteriel aktivitet er stærkt relateret til tilstedeværelsen af partikelært organisk materiale. Flow cytometri kan også med fordel anvendes til at kvantificere frit levende celler i vandfasen, som var stærkt korreleret med den tilgængelige opløste fraktion af det organiske stof.

Denne undersøgelse har vist nye aspekter af bakteriel dynamik i RAS ved under kontrollerede betingelser at måle bakterier i vandfasen. Det anbefales, at der i fremtidige undersøgelser fortsættes med at belyse: i) biofilters mikrobielle sammensætning i forhold til bakterier i vandfasen (frit levende bakterier og partikelbundne bakterier); og ii) fritlevende bakteriesamfund i forhold til partikelassocierede bakteriesamfund.

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List of Abbreviations

AOB	: Ammonia oxidizing bacteria
AOC	: Assimilable organic carbon ATP: Adenosine tri-phosphate
BOD ₅	: Biological oxygen demand of five days
BDOC	: Biodegradable dissolved organic carbon
CFB	: cumulative feeding burden
C/N	: carbon to nitrogen ratio
COD	: Chemical oxygen demand
DGGE	: Denaturing gradient gel electrophoresis
DOC	: Dissolved organic carbon
FCM	: Flow cytometry
FSC	: Forward scatter
HDNA	: High DNA
HRT	: Hydraulic retention time
LDNA	: Low DNA
MUW	: Make-up water
MWQ	: Microbial water quality
NOB	: Nitrite oxidizing bacteria
PAB	: Particle-associated bacteria
PB	: Planktonic bacteria
PCR	: Polymerase chain reaction
RAS	: Recirculating aquaculture system
SSC	: Side scatter
TAN	: Total ammonia nitrogen
TOC	: Total organic carbon
UV	: Ultraviolet

Introduction

Water quality can be assessed according to chemical, physical and microbiological characteristics (Kirmeyer et al., 2001). As chemical and physical parameters characterize the physicochemical water quality, microbial water quality involves the measurement of microorganisms as e.g. bacteria, algae, protozoa and other organisms in water (Gerardi et al., 2016). The assessment of microbial water quality can be done in function of the microorganism's abundance, viability, activity, and/or composition and structure of the microbial community (Attramadal et al., 2012b, Arvin and Pedersen, 2015; Prest et al., 2016a). In recirculating aquaculture systems (RAS), chemical water quality management is well established (Bergheim et al., 2009; Timmons et al., 2009; Dalsgaard et al., 2013). Microbial water quality criteria do not exist and presently there are no well-defined suites of parameters/assays to be monitored.

The main interest in microbial water quality within RAS is due to the constant challenge caused by high organic loads/input. Bacteria have a key role in a number of the different biological processes in RAS (Blancheton et al., 2013; Rurangwa and Verdegem, 2015). Several factors affects these processes and regulate bacterial growth such as availability of organic and inorganic nutrients (Avnimelech, 1999; Zhu and Chen, 2001; Leonard et al., 2002; Michaud et al., 2006; **Paper III**), environmental conditions such as pH, oxygen and temperature (Zhu and Chen, 2002; Salvetti et al., 2006; Kinyage and Pedersen, 2016; Prest et al., 2016a), spatial availability as e.g. particles, water column, sediment or biofilm (McDougald et al., 2011; Fernandes et al., 2017; Pedersen et al., 2017) and presence of predators/phages e.g. protozoa and other invertebrates (Hahn and Höfle, 2001). System treatments units, management and water distribution conditions (e.g. hydraulic retention time and/or plumbing setup) affect each of these factors and contributes to shaping the bacterial community characteristics (Nogueira et al., 2002; Schneider et al., 2007; Attramadal et al., 2014).

Two important groups of bacteria exist within RAS. These are the autotrophic nitrifiers that oxidize ammonia to nitrate (Hagopian & Riley) and heterotrophic bacteria that degrade organic matter (Metcalf and Eddy Inc. and Tchobanoglous, 2003). In an ecological sense, most heterotrophic bacteria are "neutral microbes" which contribute to microbial water quality by using resources and preventing the establishment of harmful species (Vadstein et al., 2004; Attramadal et al., 2012a,b; Blancheton et al., 2013). Heterotroph's five-fold higher growth rate in comparison to the autotrophic bacteria (Ebeling et al., 2006) can be a disadvantage when systems face abrupt changes in organic load, where they can out-compete the autotrophs and thereby affect the biofilter performance or compete for oxygen with the actual species being farmed. Among the heterotrophic fast-growing (opportunistic) bacteria, detrimental and pathogenic species might be found (Allen et al., 2004). Unwanted changes in microbial water quality in RAS could have adverse effects on the species reared (Moestrup et al., 2014), therefore constant conditions as opposed to fluctuating conditions could enhance biological stability (stable matured water) (Attramadal et al., 2012a, Prest et al., 2016a). This can only be managed by controlling chemical and microbial water quality in line with a proper RAS management.

Recently, aquaculture industry development is expanding towards intensive RAS. Here, new challenges are met in relation to water quality management due to the restricted use of make-up water which leads to accumulation of waste products, proportionate to the large production volumes (Martins et al., 2010, Dalsgaard et al., 2013, Verdegem, 2013). For this reason, microbial control should be regarded as another

central management factor/tool to be developed. Research on microbial water quality within RAS has been progressing slowly due to time-consuming, non-informative and/or complex assays, and simple and reliable measurement/monitoring systems are needed. Therefore, there is no clear data knowledge and no empirical knowledge base or consensus on which parameters should be measured. If such data existed, they could eventually be integrated into a toolbox and help to establish guidelines to define “good” or “acceptable” levels of microbial water quality for the rearing organism in question.

Over the last decades, a number of methods to assess the bacterial growth-supporting properties of water have been developed to support water utilities (Vital et. al., 2012; Prest et al., 2016a). Now, focus has been set on the development of rapid cultivation-independent methods for bacterial enumeration that enables accurate and fast analysis of the general microbiology water quality (Berney et al., 2008; Hammes et al., 2008, 2010; Reeslev et al., 2011; Besmer and Hammes, 2016; Højris et al., 2016). The application of methodologies with these characteristics within RAS management could contribute substantially to detect abrupt system changes within short laps of time and thereby enable fast decision-making in case of e.g. imbalances, malfunctioning of a treatment devices or accidental mismanagement.

The following sections will give an outline on different possible monitoring tools for assessing microbial water quality within RAS. In addition, an overview based on the existing knowledge regarding microbial water quality in RAS will be given, focusing on factors that affect bacterial dynamics such as: organic and inorganic nutrient fluxes, environmental parameters, feed loading, treatment components, management, and others.

Objectives

The overall objective of this thesis was to assess microbial water quality in freshwater recirculating aquaculture systems (RAS). Establishment of new Recirculating aquaculture systems is taking place at a high pace these years and there is an urgent need to understand factors that affect bacterial growth in RAS water. New assessment tools are being developed and implemented which focus on reliable and rapid methods to assess bacterial activity and abundance in water. In this project, controlled and prolonged experiments in replicated RAS with rainbow trout (*Oncorhynchus mykiss*) were established as the basic starting point to provide new knowledge on the causal relationship between production factors and microbial water quality. The aim of this thesis was to investigate additional water quality parameters in relation the bacterial abundance and activity in RAS water tested in replicated RAS, reflecting realistic production conditions with particular emphasis on; oxygen, temperature, pH, alkalinity, flows, feed-loading and water exchange. Three experimental series were performed as follows:

- 1) Investigation of microbial water quality during start-up of a RAS (**Paper I**). The aim of this study was to monitor the dynamic of microbial water quality by measuring bacterial activity from the start-up of RAS over a three months period. A new methodology “Bactiquant®” (for assessing bacterial activity in water) was applied in combination with a set of chemical water quality parameters and fish performance. Thus, assess the microbial succession from varying to more stable conditions.
- 2) Studying microbial water quality in RAS associated with abrupt changes in feeding loading (**Paper II**). The aim of this investigation was to study and evaluate microbial water quality changes in RAS at three distinct levels of feed loading. This was experimentally done by establishing three treatment groups with: i) no feed; ii) unchanged; and iii) doubling feeding. The microbial water quality was assessed in terms of bacterial activity and abundance for a successive period of 7 weeks. Bacterial activity was measured with the Bactiquant method and a new method “hydrogen peroxide (HP) degradation assay” and abundance of free-living bacteria was analyzed using flow cytometry. The experiment included six identical and previously “conditioned” RAS and chemical parameters were included as part of the assessment and for further comparison with microbial water quality parameters.
- 3) Evaluation of potential changes in RAS microbial water quality associated with the addition of a dissolved, easily biodegradable carbon source (**Paper III**). The aim of this study was to assess the abrupt changes on selected microbial water quality markers in RAS with the monitoring tools already used in **Paper II**. In this prolonged and controlled experiment, twelve identical and independent RAS (previously conditioned) were used. The potential interaction between biofilters and bacteria in the water phase was also considered.

Microbial Water Quality in RAS

1. Recirculating aquaculture systems

Recirculating aquaculture systems (RAS) have emerged as a potential solution to solve conflicting interest such as environmental restrictions and claims on water sources with an increasing demand for the production of fish. Due to the capacity of these systems to provide good and controlled rearing conditions, RAS have gained considerable interest and have proliferated in recent years. Recirculating aquaculture systems (Fig. 1) reuse the water as opposed to traditional aquaculture systems (Michaud et al., 2014) by treating the rearing water (Martins et al., 2010). RAS also present other important benefits such as temperature control, supporting controlled and more reliable production strategies, full control of water quality, biosecurity control and disease management, and reduction of environmental impact and risk of fish escape (Timmons et al., 2009).

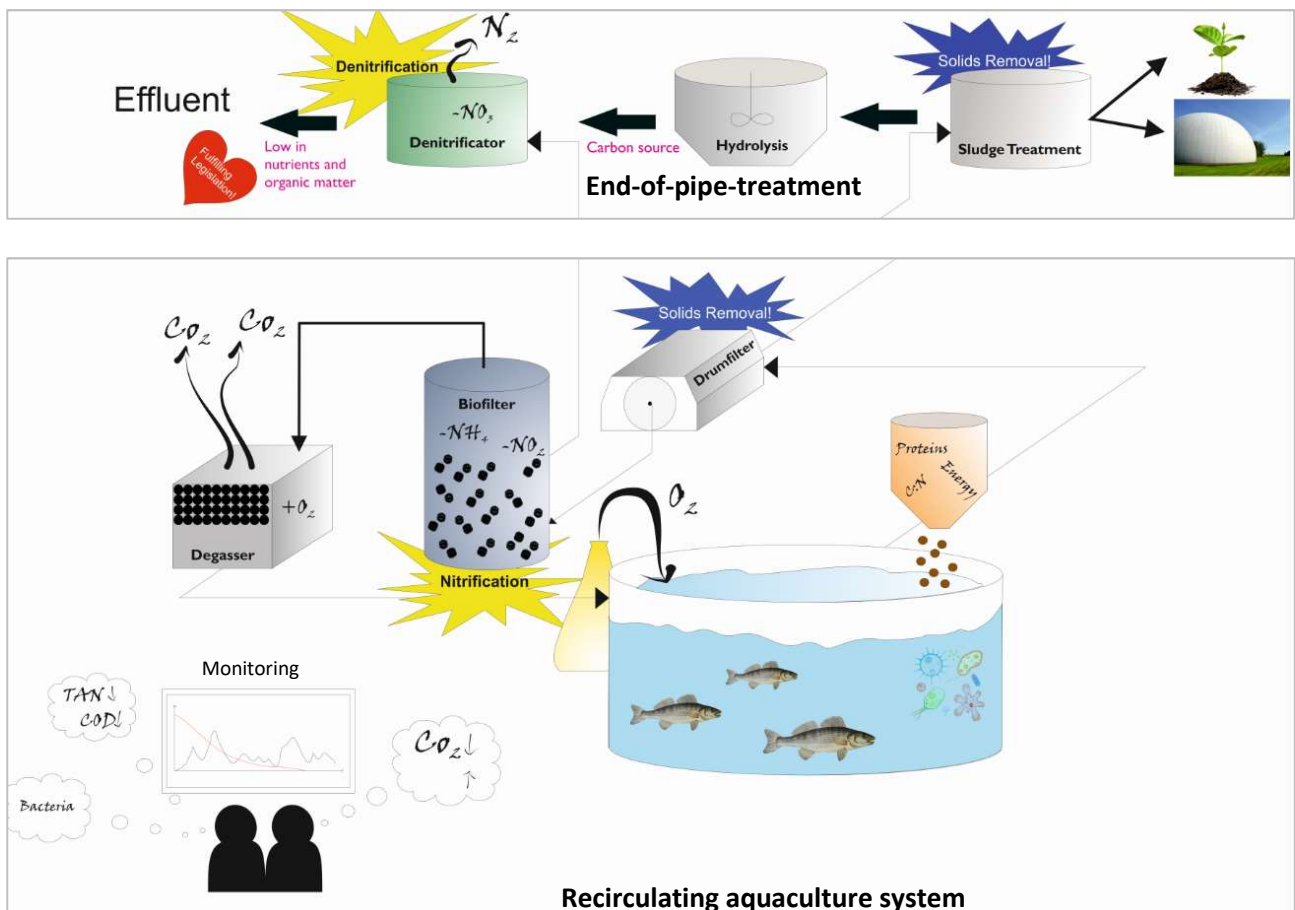


Fig. 1: Basic components of a recirculating aquaculture system with end-of-pipe treatments.

Recirculating aquaculture systems are classified according to their degree of water recirculation but also according to feed loading (also called cumulative feeding burden - CFB) defined as kg of feed applied per m³ of make-up water (MUW) added, and is used as a measure to describe the production intensity of the overall system (Colt et al., 2006; Pedersen et al., 2012b). As intake water is reduced, waste accumulates within the systems and more treatment units are then required to maintain proper water quality (Fig. 2).

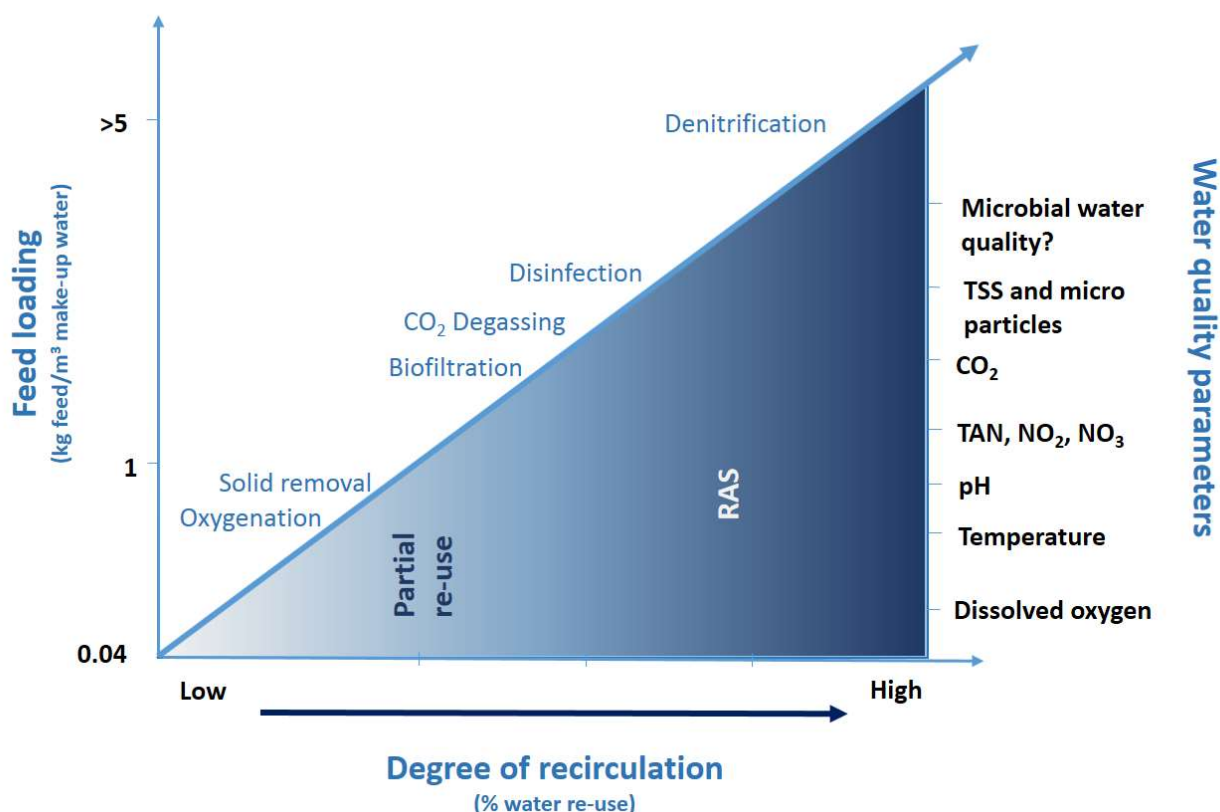


Fig. 2: Conceptual relation between degree of recirculation and treatment units, feeding load and importance of water quality parameters (Modified from Fernandes, 2015).

Land-based aquaculture systems can be classified according to their feed loading as: flow-through (< 0.04 kg feed/m³ MUW), partial re-use (0.04 - 1 kg feed/m³ MUW) ranging from 0 to 70% re-use, conventional recirculation (1 - 5 kg feed/m³ MUW) with 70 to 95% recycling and innovative RAS (5 kg feed/m³ MUW) with >97% re-use (Martins et al., 2010).

Recently, RAS for the full-life/production cycle of a species have appeared. In Denmark alone, there are four companies producing between 600 and 1000 tons/year of fish in RAS, operating at feed loadings between 2 to 10 kg/m³ (Table 1). At these levels of intensive RAS, proper design of treatment units and water quality are of the high importance.

Table 1: Feed loading data from actual operating RAS in Denmark and Norway.

Company	Specie	Water source	Feed loading (kg/m ³)	Production (ton/year)	Country	Reference ¹
AquaPri A/S	Pike perch	Freshwater	3 - 10	600	Denmark	Pers. Obs.
RAS2020 concept	Trout, salmon, yellowtail kingfish	Freshwater /Saltwater	3 – 6 ^a	1200	-	[1]
Langsand Laks	Salmon 3.5-4 kg	Saltwater	1-4	800	Denmark	[2]
Danish Salmon	Salmon 3-5 kg	Saltwater	2.0	700	Denmark	[3]
Lerøy Midt AS	Smolt 80 g	Freshwater	1.7	14 mill. smolt	Norway	[4]

^a Referred to design parameters of RAS2020 concept. Three companies are producing fish with RAS2020 design: Swiss Alpine Fish in Switzerland, and Fredrikstad Seafoods in Norway (upcoming), and Sashimi Royal in Denmark

[1]: Veolia RAS2020; [2]: Billund Aquaculture AS; [3]: Danish Salmon A/S; [4]: Lerøy Midts AS.

In RAS, the main waste source is fish metabolites and any uneaten feed (Timmons et al., 2009). Several treatment steps are required to handle the waste (Fig. 1 and 2) and can be overall described in the following order:

- 1) The first treatment step is removal of solid organic matter. There are several ways to remove solid organic matter but the most common device in commercial RAS are rotating micro screens or “drum filters”. The micro screen used in drum filters is normally for removing solids larger than 40 – 100 µm and a major advantage is their high flow filtration capacity (Fernandes et al., 2015).
- 2) The second step is the conversion of ammonia (NH₃) into nitrate (NO₃⁻). This nitrification process is performed inside biofilter units.
- 3) The third step consist on water passing by a degassing/aeration unit. Here, CO₂ is removed and some oxygen is incorporated into the water.

Generally, all these treatment steps are carefully monitored by a set of water quality parameters.

Additional treatments such as disinfection are implemented according to the needs of each farm and can be applied as preventive measures (Torgersen and Håstein, 1995; Lekang, 2007; Timmons et al., 2009). Ultra violet irradiation is the most common disinfection unit used, as well as ozone (Bullock et al., 1997; Liltved and Cripps; 1999; Tango and Gagnon, 2003; Sharrer et al., 2005; Sharrer and Summerfelt, 2007; Summerfelt et al., 2009; Powell et al., 2015).

Denitrification treatment units as end-of-pipe treatment (Fig. 1) are used to decrease the load of N-compounds being released into the environment and e.g. in Denmark strict restrictions are applied (Letelier-Gordo et al., 2014). Systems operating at high feed loadings often should consider the application of denitrification within the recirculation loop in order to reduce the NO₃ concentration in the recycled water loop.

A major challenge to RAS water quality is the potential impacts of fine solids that are not removed in the primary treatment (Colt, 2006). In terms of the rearing species, there are on-going discussions whether particles directly affect fish health or not (Becke et al., 2017, Chapman et al., 1987; Bullock et al., 1994; Lake and Hinch, 1999; Au et al., 2004). From a chemical and microbial water quality point of view particles are critical. Studies have shown that 95% of the suspended solids consist of fine particles below 20 μm (Chen et al., 1993; Fernandes et al., 2015). The surface area of these particles serve as habitat for bacteria to attach to as well as substrate for further growth (Pedersen et al., 2017). Particles will continuously be degraded until eventually becoming part of the dissolved fraction and by that become substrate for free-living (also called planktonic) bacteria (further discussed in section 3.1). Hence, the microbial state of RAS water depends on the supply of bacteria and organic matter with the consideration of the selective forces in the rearing tank (Attramadal et al., 2012 a,b; Wold et al., 2014).

Microbial water quality has been of interest for larvae/juvenile performance in RAS for years due to the detrimental but also the potentially beneficial effects bacteria can have on these vulnerable life stages (Vadstein, 1997; Skjermo et al., 1997; Attramadal et al., 2012a). In later stages, fish are considered more robust and other requirements i.e. oxygen demand and solid removal, becomes more important. Nevertheless, problems in Danish RAS operating at feed loadings above 2 kg/m^3 , have experienced changes in nutrient flows and increased microbial abundance and activity causing near-catastrophic events in RAS (pers. obs.; Moestrup et al., 2014). It seems that fluctuations in microbial water quality could become critical even for large fish. This also suggests that there is a direct need for application of tools to rapidly assess microbial water quality.

2. Assessment of microbial water quality

Generally in water utilities, microbial presence and activity is paradoxically viewed as either beneficial (e.g. organic and inorganic nutrients consumption in biofilters) or negative (e.g. increase in turbidity, biofouling, growth of opportunistic organisms) (Vital *et al.*, 2012). In both cases, correct qualitative and quantitative information regarding bacterial concentration and composition is important for detecting specific events and to understand and control treatments processes.

Operational factors, such as inadequate removal of unconsumed feed and fecal matter, contribute to the build-up of organic matter within RAS (Wold *et al.*, 2014; Hambly *et al.*, 2015; Fernandes *et al.*, 2015) and might affect microbial water quality. Disinfection (Attramadal *et al.*, 2012b), changes in feed composition and feed digestibility (Lam *et al.*, 2008), recirculation rates and water exchange volumes (Blancheton *et al.*, 2013; Rurangwa and Verdegem, 2015) and feed loading (Pedersen *et al.*, 2012b; von Ahnen *et al.*, 2015; **Paper II**) may also contribute to the destabilization of microbial water quality.

There are three main ways to assess microbial water quality in RAS:

- 1) simple observations of water deterioration (e.g. turbidity or color of water) and fish performance;
- 2) the use of predictive methods that involves indirect measurements based on the amount of organic matter in the water;
- 3) the application of direct methods that can detect changes in bacterial abundance, activity/viability and/or community composition.

The following sections will present a brief overview of the different predictive and direct methods used for assessing microbial water quality in RAS. Only recently, microbial water quality monitoring systems/methods have received attention and significant progress in the technology have been made (Fig. 3).

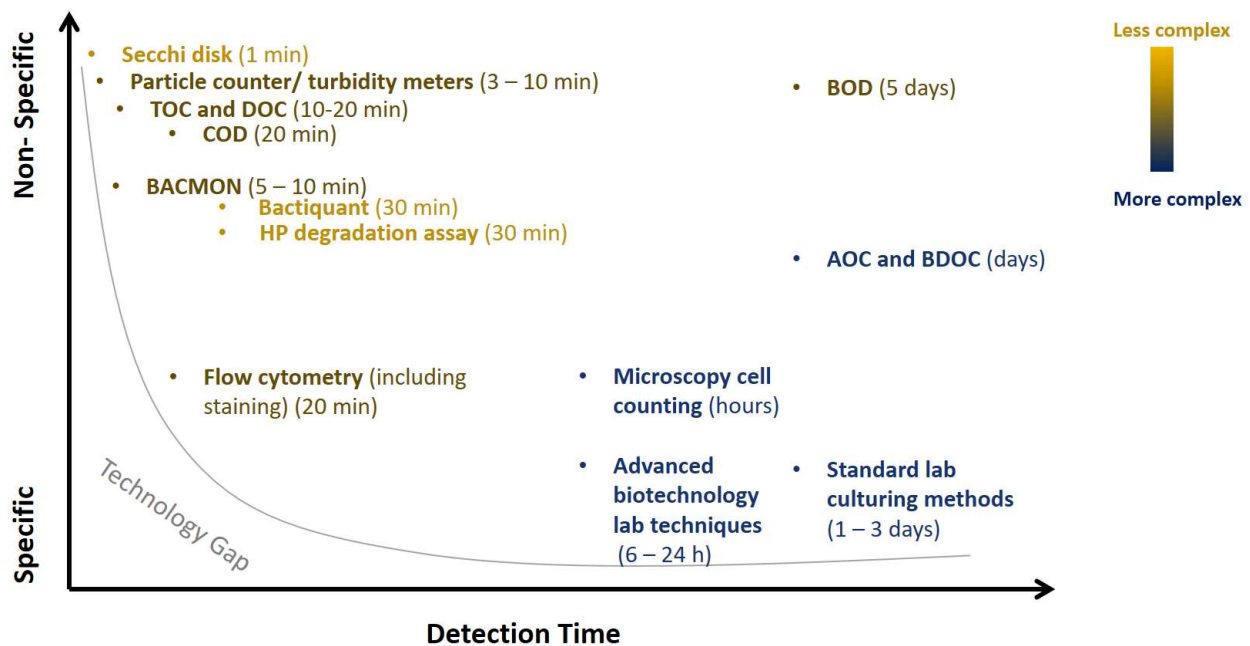


Fig. 3: Overview and comparison of methods in relation to their detection time, specificity and complexity (Total organic carbon (TOC), dissolved organic carbon (DOC), chemical oxygen demand (COD), biological oxygen demand (BOD), assimilable organic carbon (AOC), Biodegradable dissolved organic carbon (BDOC)). (Modified from Grundfos AS).

2.1 Predictive method

Several strategies based on the quantification of organic matter related parameters are used to predict bacterial load in the water. Organic matter contains organic and inorganic nutrients that sustain microbial growth, predominately heterotrophic growth (Blancheton et al., 2013). Organic matter is composed by a matrix of different organic compounds that can be assimilated by the bacteria. Therefore, different collective analyses that include different fractions of the organic matter can be used (Table 2).

Table 2: Outline of different methods for assessing organic matter in water.

Method	Measures	Principle	Labor	Time-to-result	Online	References ¹
Biochemical oxygen demand (BOD ₅)	Biodegradable organic matter	Bacterial respiration (oxidation)	High	5 days	Yes	[1], [2]
Chemical oxygen demand (COD)	Organic matter	Chemical Oxidation	Low	30 min	Yes	[1], [2]
Total organic carbon (TOC)	Total organic carbon	Oxidation	Low	5-10 min	Yes	[1]
Dissolved organic carbon (DOC)	Dissolved organic carbon	Oxidation/UV absorbance 254 nm	Low	5-10 min	Yes	[1], [3]
Total and Volatile suspended solids (TSS/VSS)	Volatilized organic matter	Gravimetric	High	24 hours	No	[1], [2]
Turbidity	Suspended organic matter	Light transmittance	Low	Minutes	Yes	[1]
Optical absorbance	Suspended organic matter	Absorbance - 600nm	Low	Seconds	Yes	[1]
Fluorescence	Dissolved organic compounds	Fluorescence	Low	Minutes	No	[3], [4], [5]

[1]: Metcalf and Eddy Inc. and Tchobanoglous, 2003; [2]: Henze et al., 1997; [3]: Hambly et al., 2015; [4]: Spiliotopoulou et al., 2017; [5]: Yamin et al., 2017.

The most common and current methods to assess organic matter in aquatic environments are gross indicators such as the biochemical oxygen demand (BOD₅), chemical oxygen demand (COD) or turbidity (Table 2). The BOD₅ assess the readily biodegradable fraction of the organic matter in RAS water (Dalsgaard and Pedersen, 2011) measured as oxygen consumption due to microbial degradation of the organic matter in five days. The chemical oxygen demand is the oxygen needed for total chemical oxidation of the organic matter in a sample.

Both total BOD₅ and COD (BOD_{TOT} and COD_{TOT}) comprise the total content of organic matter but can be subdivided into a particulate and a dissolved fraction (COD_{PART} and COD_{DISS}). The COD_{DISS} fraction is obtained from a sample filtered through a 0.45 µm membrane and COD_{PART} is then calculated in the following way: COD_{PART} = COD_{TOT} - COD_{DISS}. In wastewater treatment, the COD_{PART} is frequently used to assess biomass development of activated sludge (Münch and Pollard, 1997; Contreras et al., 2002). During this thesis study, COD_{DISS} measurement was modified by filtering the sample through a smaller pore size of 0.2 µm. This was done due to the bacteria size-range of 0.2- 3 µm (Gerardi, 2006) and to make sure that COD_{PART} included the whole fraction of the biomass. By doing this change in the COD_{DISS} analysis, good correlations were obtained when comparing COD_{DISS} to DOC (Fig. 4) from same RAS water samples collected from the start-up to steady-state conditions of 6 identical RAS operating at different feed loading (**Paper I and II**). At the same time, values of COD_{PART} showed good correlation with bacterial activity (Bactiquant®) as well (Fig. 5).

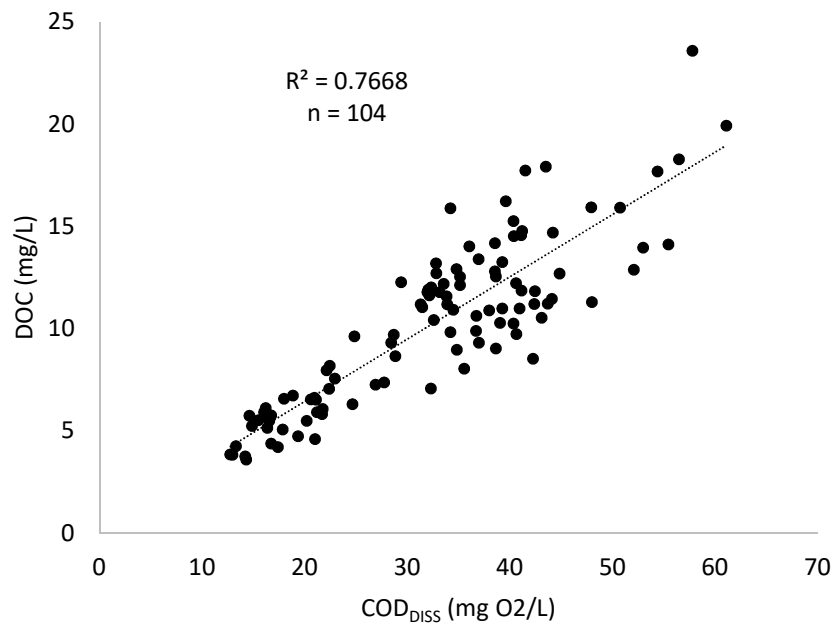


Fig. 4: Correlation between DOC (samples filtered through 0.45 μm) and COD_{DISS} (samples filtered through 0.2 μm), both parameters from same RAS water samples (Data not published).

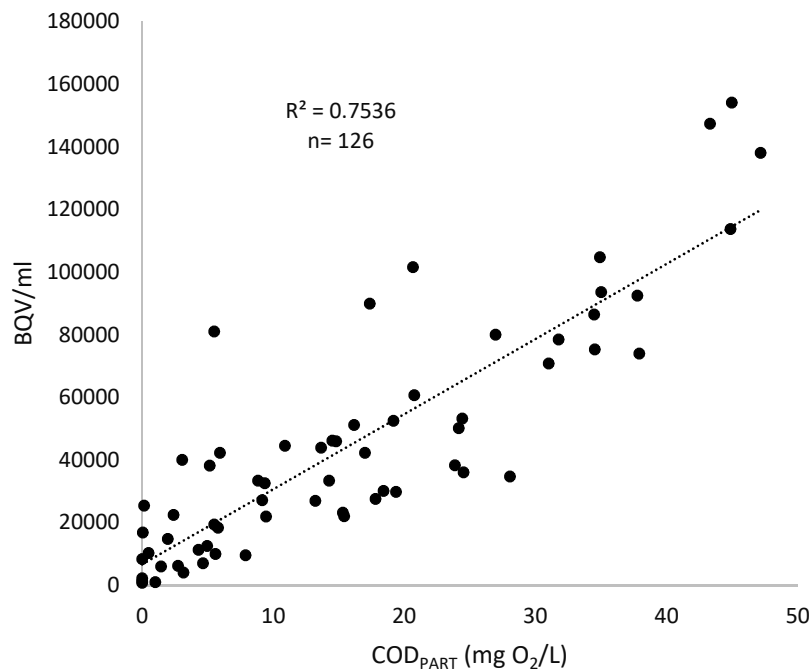


Fig. 5: Correlation between bacterial activity (Bactiquant®) and particulate COD (COD_{PART}). Bactiquant® is a patented method that assess bacterial activity indirectly by targeting an enzyme from the hydrolase class. The method measures bacterial activity in a unique value called bactiquant value (BQV) (unpublished data).

Given the nature of the analysis, COD will always be higher than the BOD since some compounds will not biologically degraded within 5 days and some can only be oxidized chemically. Interrelationship between

BOD and COD, (and also TOC; Table 2) are done to understand the degradability/fractionation of the organic matter in the water (biodegradability index are discussed further in section 3.2.2)

The advantage of COD compared to BOD₅ is the analysis duration (Fig. 3, Table 2). The COD analysis with commercially available kit offers results within minutes. However, this procedure may become less economically feasible when many samples need to be analyzed and it produces toxic waste that needs to be handled properly. BOD₅ analysis is considered simple but it takes time to prepare samples and the incubation period is 5 days. This makes it more-or-less unusable for monitoring an operational system.

Of the accumulated organic matter within RAS, only a small portion of the organic carbon remaining in the water is available as a source of carbon and energy for microorganisms (van der Kooij et al., 1982). In RAS, carbon is considered the limiting factor for heterotrophic growth (Leonard et al., 2002, **paper II**). Despite the complexity of the carbon matrix and the difficulties associated with determining the different fractions, some efforts have been done on this aspect.

van der Kooij et al. (1982), suggested a method for determining the assimilable organic carbon (AOC). The assay focuses on the determination of easily available substrate for planktonic growth, by measuring the growth of a specific bacterium in the water sample. Later, Servais et al. (1989) introduced another method based on measuring biodegradable dissolved organic matter (BDOC). The BDOC assay consists of filtering the water sample through a 0.2 µm pore size membrane and then inoculate it with an autochthonous bacterial population. Finally, the decrease in DOC concentration due to carbon oxidization by bacteria is measured. Both methods describe the potential of the water for supporting microbial regrowth and have with time, been adapted and improved in relation to their representativeness, easy handling and test timing (Prest et al., 2016a). These methods are useful for getting a better understanding of the dynamics between organic matter and planktonic bacteria within RAS on a research basis but seem not suitable for RAS operational management.

Turbidity is probably the most used method to assess particulate organic matter levels within RAS. It can be measured by a turbidity-meter or simply by a Secchi disk. Some farms have managed to establish a daily routine measuring turbidity and associated increase in turbidity to bacterial growth, and thereby they are aware of potential changes which allow corresponding actions as for example the activation of a corresponding disinfection unit.

2.2 Direct methods

2.2.1 Bacterial abundance

Traditional plate counting on agar is a classic culture-dependent technique to evaluate bacterial numbers (Atlas and Bartha, 1998). The main drawback of this method is the low representability since less than 1% of the microbial species are actually able to grow under standard laboratory conditions. In addition, the method is quite time-consuming. In the drinking water sector, plate counting on agar is the only method incorporated in the legislation (e.g. máx. value: 20-500 CFU/ml) (Van Nevel et al., 2017). Within aquaculture, this is not a routine measurement. Despite the drawbacks mentioned, a wide range of studies have used this approach for detecting and/or quantifying bacteria in aquaculture water (Table 3).

Table 3: Overview of different direct methods used for bacterial quantification.

Method	Measures	Principle	Viability	Labor	Time-to-result	Online	References ¹	Applications in RAS studies ¹
Plate count agar	Cultivable bacteria	Growth	Yes	Medium	Days to weeks	No	[1], [2]	[14] to [25]
Microscopy	Cell concentration	Staining	Yes	High	Minutes to hours	No	[3]	[19], [23], [21], [22], [26]
Flow cytometry	Cell concentration	Staining	Yes	Low	Minutes	Yes	[4], [5], [6], [7], [8]	[27], [28], [29], [30]
ATP	ATP concentration	Enzymatic	Yes	Low	Minutes	Yes	[9], [10], [11]	-
Bactiquant®	Fluorescence	Enzymatic	Yes	Low	Minutes	No	[12]	[31], [32], [33]
HP Assay	Absorbance	Enzymatic	Yes	Low	One hour	No	[13]	[34]
Particle count method	Particles concentration	Coulter counter	No	Low	Minutes	Yes	[36]	[35]

[1]: Allen et al., 2004; [2]: Van Nevel et al., 2017; [3]: Cragg and Parkes; 2014; [4]: Troussellier et al., 1993; [5]: Vives-Rego et al., 2000; [6]: Wang et al., 2010; [7]: Besmer et al., 2014; [8]: Besmer and Hammes, 2016; [9]: Berney et al., 2008; [10]: Hammes et al, 2010 [11]: Vang et al., 2014; [12]: Reeslev et al., 2011; [13]: Arvin and Pedersen, 2015; [14]: Blancheton and Canaguier, 1995; [15]: Leonard et al, 2000; [16]: Salvesen and Vadstein, 2000; [17]: Sharrer et al., 2005; [18]: King et al., 2006; [19]: Michaud et al., 2006; [20]: Sharrer and Summerfelt, 2007; [21]: Attramadal et al., 2012a, [22]: Attramadal et al., 2012b; [23]: Michaud et al., 2014; [24]: Garrido-Pereira et al 2013; [25]: Fu et al., 2015; Powell et al., 2015; [26]: Wietz et al., 2009; [27]: van der Meer, 2011; [28]: Attramadal et al., 2014; [29]: Attramadal et al., 2016; [30]: Wold et al., 2014; [31]: Fernandes, 2015; [32]: Pedersen et al., 2017; [33]: Paper I; [34]: Paper II; [35]: Paper III; [36]: Coulter principle short course, 2014.

A number of quantitative and molecular methods (e.g. flow cytometry) have appeared recently. They allow the evaluation of microbial diversity, provide information regarding their function and are thereby leading to a better understanding of the interaction within communities (Schreier et al., 2010; Van Nevel et al., 2017; Table 3). Quantitative methods, such as direct counting in a microscope using for example fluorescent diamidino-2-phenylindole (DAPI) (Michaud et al., 2006, 2014; Attramadal et al., 2012a,b), acridine orange direct count (AODC) staining (Cragg and Parkes; 2014), fluorescents in situ hybridization (FISH) (Wietz et al., 2009) have been used in some studies to evaluate the bacterial load in water. All these methods are specific and reliable but some may be challenging for use in aquaculture monitoring as they are time consuming and generally too expensive for microbial water quality monitoring on commercial farms.

Flow cytometry (FCM) is a method that counts cells rapidly within 20 min (inclusive of the required staining time) with high precision (Vives-Rego et al., 2000). Cells are stained with a fluorescent molecule (Wang et al., 2010) and the instrument can exclude inert particles (Fig. 6). This counting method has been used for

characterization and quantification of microbes in natural aquatic habitats for several decades (Troussellier et al., 1993; Wang et al., 2010) but only recently it was introduced as a method for drinking water analysis (Van Nevel et al., 2017). Recent aquaculture studies have used FCM for quantifying bacterial abundance in marine larvae RAS water (van der Meeren, 2011; Attramadal et al., 2014, 2016; Wold et al., 2014) and in freshwater RAS (**Paper I and II**).

The flow cytometer instrument consist of three main components (Brandsegg, 2015; Fig. 6):

- Fluids: Transports the cells to the laser beam in a narrow stream.
- Optics: The lasers illuminates the cells and filtrate the light signals directly to the detectors.
- Electronics: The detected light signals are converted into electrical signals that further processed in a computer.

Once the cell suspension is inside the flow cytometer, an isotonic fluid creates a laminar flow and the cells align into a narrow stream. Each single cells passes by a powerful light source, which is often a laser, and scatter of light occurs each time a cell passes. The shape, size and refractive indexes of the cell defines the angular intensity. When cells are stained with a fluorescent dyer that comprise an absorption spectrum that corresponds to the laser – the excitation source- then the laser excites the fluorescent molecules to a greater energy state (Marie et al., 2005).

The light that is emitted from all the directions as the cell passes by the laser, is collected by optics and conducted to filters. The filter together with the dichroic mirrors sends the light signals to the detectors that will collect light at specific wavelength (Brown and Wittwer, 2000)

The different light detectors indicates different features of the cell passing by the light source. The forward-scatter's (FSC) light is proportional to size of the cell or its cell-surface area. Moreover, the side-scatter's (SSC) light reflects the cell surface and internal cellular structures as e.g. granularity (Marie et al., 2005; Brandsegg, 2015).

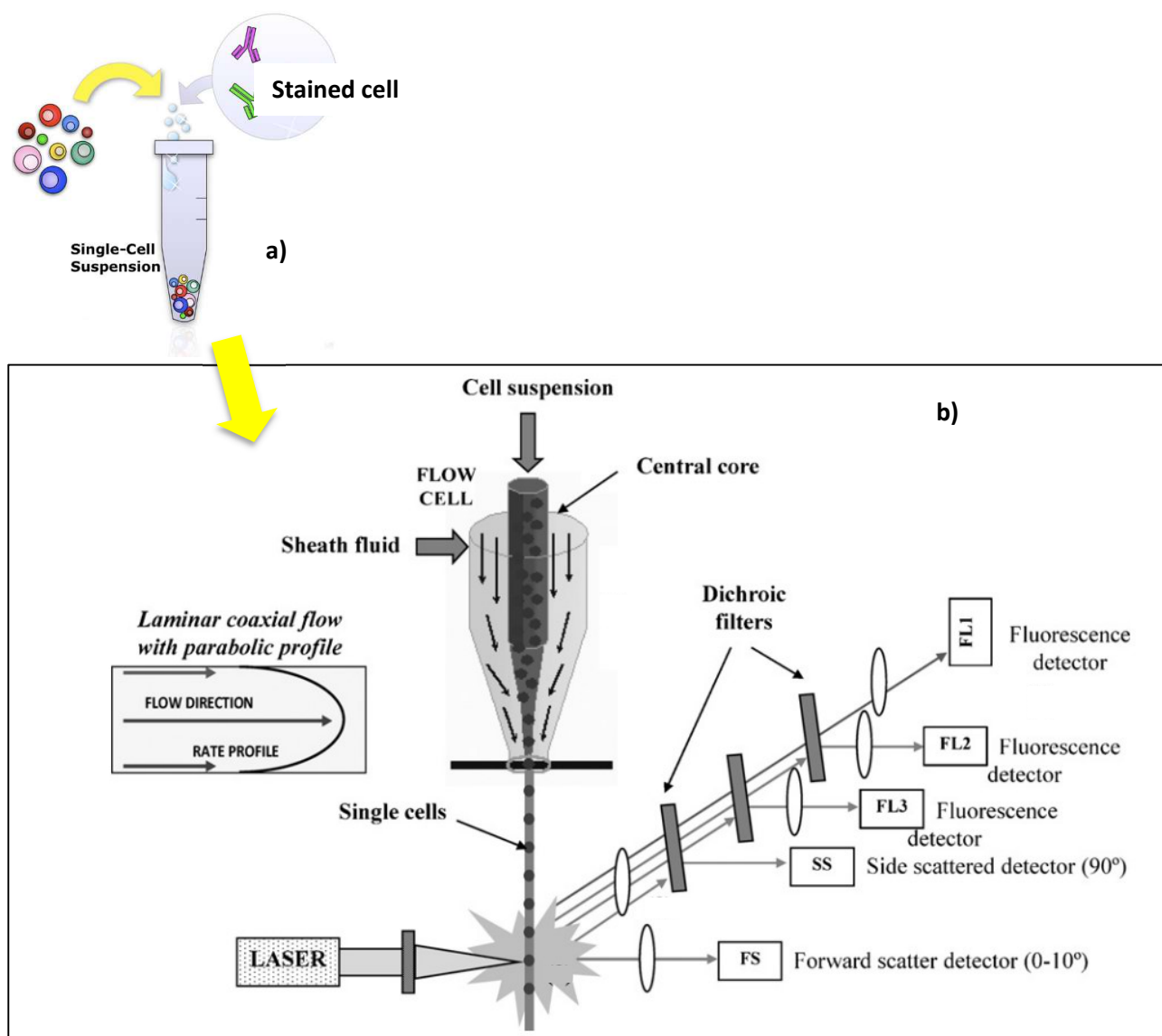


Fig. 6: Schematic overview of cell staining and components of flow cytometer (FCM): a) Bacterial cells in a water are stained with a fluorescent molecule before entering the FCM; b) Cell suspension is put into the flow cell where cells are aligned one by one. Once the cell passes by the laser, this emits a light that is further collected by the different detectors (From: Díaz et al., 2010).

Besides determining total and intact cell concentration, another qualitative value of FCM is the capability of creating a more detailed analysis called “microbiological fingerprints”. Microbial fingerprints are statistical analyses of the information obtained from the fluorescence and scatter detectors. These FCM data are related to e.g. cell size, fluorescent color, and fluorescence intensity and are represented in the distribution of the raw data in the FCM plots. The advantage of the FCM fingerprinting method is that it is sensitive towards detection of small changes and shifts within the bacterial population, which cannot be noticed using enumeration only.

As an example, Fig. 7 depicts FCM plots from RAS water samples showing the fingerprint of the water. Water samples collected weekly for ten weeks shows the changes of cell clusters over time, revealing the dynamics

of cell abundances in the water. These clusters have been distinct in other studies and defined as high-DNA (HDNA) bacteria and low-DNA (LDNA) bacteria referring to the difference in the fluorescence intensity between groups (Gasol et al., 1999; Lebaron et al., 2001). The formation of a high DNA (HDNA) cell cluster in Fig. 8 are observed from week zero, reaching the highest development in week six and then starting to disintegrate again. There are no published studies indicating the development of this dynamic within RAS water, but the HDNA cell clusters have been observed in water samples from different aquatic environments (Gasol et al., 1999; Lebaron et al., 2001; Hammes et al., 2008; Tarran et al., 2015). High DNA bacteria are described in literature as dynamic members of the bacterial community who have a rapid response to predation pressure and nutrient availability (Gasol et al., 1999).

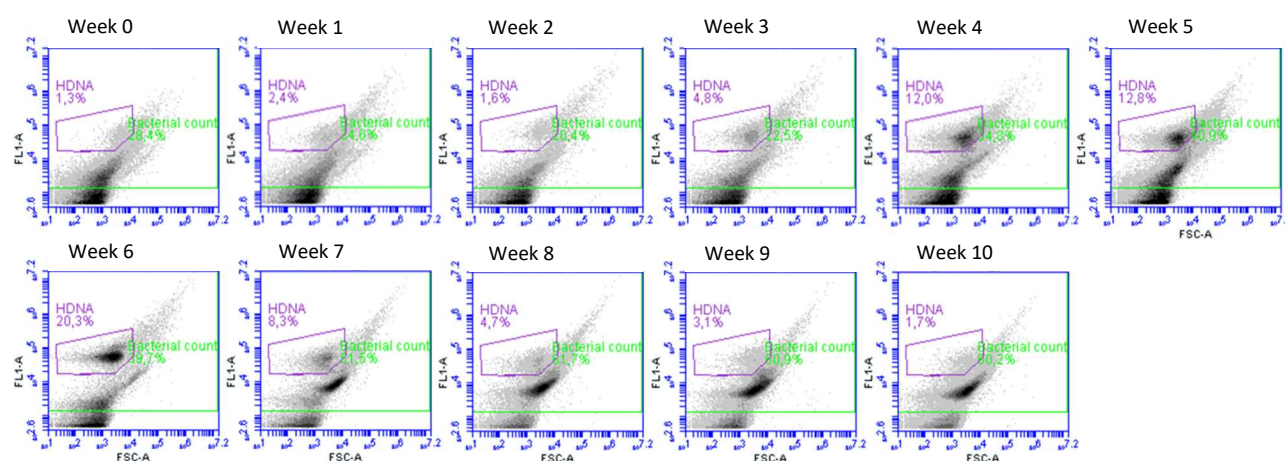


Fig. 7: Flow cytometry dot plots of water samples from a three months conditioned RAS. All samples were stained with SYBR Green I and analyzed using flow cytometry. FL1A denotes green fluorescence signals (520 nm) in y-axis and FSC- A (forward scatter) in x-axis. Purple gating - called HDNA - separate the high DNA content cells and the green gating (line is the FL1A thresholds – separating the bacterial cells from the background noise (Unpublished data).

Despite the available information, there is still no clear answer in relation to the composition and function that HDNA cells provide within the bacterial dynamics in water (Lebanon et al., 2001). Further research on this matter could provide valuable information for understanding microbial dynamics within RAS water.

Even though FCM is well suited for enumeration of suspended bacterial cells, this methodology cannot be applied directly for counting bacteria attached to particles, unless specific pre-treatment such as ultrasonication or physical removal (e.g. scrubbing or swabbing the surface) are provided to suspend the attached bacteria (Prest et al., 2016a). Flow cytometry data can - however - provide distinction between dead and live, or intact or damaged cells by the addition of proper staining dyes.

Besmer et al. (2014) showed the feasibility of automated flow cytometry and later, Besmer and Hammes (2016) managed to describe the microbial dynamics in a drinking water plant by the use of on-line flow cytometry, providing a significant jump across the technology gap (Fig. 3).

Online monitoring technologies for assessing bacterial abundance and activity in water have recently been provided by Danish companies. Grundfos has developed a technology (Bacmon), which corresponds to an online monitoring system based on a (photographic) sensor that recognizes and counts particles/bacteria in the water based on shape and patterns of light diffraction (Højris et al., 2016); Mycometer A/S has developed a manual method called Bactiquant® that measures bacterial activity in water (Reslev et al., 2011); and the

company SBT Aqua have developed “Bactobox”, a bacteria sensor based on using impedance flow cytometry. Bactobox should be able to perform real-time monitoring of bacteria and particles in drinking and process water. The technology is expected to be precise, cheap, and with little to no maintenance requirements. These technologies are being used or tried out in drinking water plants, revealing stable baselines and response to different events that single daily grab samples cannot detect (Fig. 8).

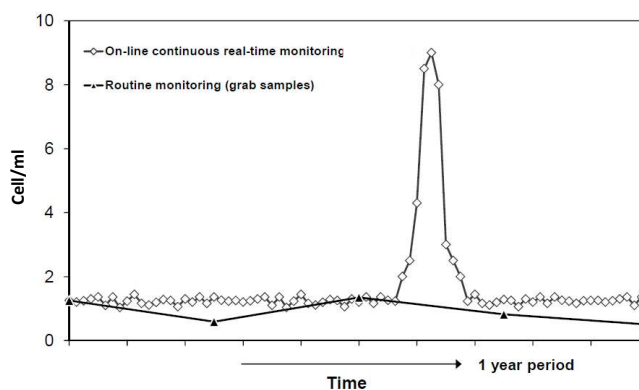


Fig. 8: Conceptual graph of contamination event detected through on-line continuous monitoring (e.g. flow cytometry) compared to occasional grab samples (Modified from Vang, 2013).

The application of potential on-line monitoring for RAS microbial water quality seems promising. Efforts have been made in relation to transfer of technology from other sectors for further use in RAS water. Some have been reasonably successful while others have been challenged. These challenges are generally related to the much higher concentration of particles in RAS water than drinking water, causing clogging or overlapping results to occur in the online monitoring systems (pers. obs).

2.2.2 Bacterial activity

Currently, at least three bacteria enzyme-based assays have become available. The cultivation-independent methods include Bactiquant® (Fernandes, 2015; Pedersen et al., 2017; **Paper I and II**), hydrogen peroxide (HP) degradation assay (**Paper I and II**), and adenosine tri-phosphate (ATP) assay (Berney et al., 2008; Hammes et al, 2010; Vang et al., 2014) are used to assess activity. All methods provide a proxy/value, which is relevant in aquatic environments such as RAS, since they comprise the activity of both particle-associated bacteria and free-living bacteria (Vital et al., 2012).

Bactiquant® is a patented method developed by Mycometer AS (Hillerød, DK). This method quantifies bacterial activity indirectly by targeting a common hydrolase enzyme found within a wide range of bacteria (Reeslev et al., 2011). A water sample of 10-50 ml is filtered through a 0.22 µm filter, on which particle-associated bacteria and free-living bacteria are retained, the filter is then exposed to a fluorescent substrate (10-30 minutes) resulting in a quantitative fluorescent signal that will indicate the bacterial load according to their activity. The results are expressed in a unique unit called Bactiquant value (BQV), calculated on the fluorescence value obtained, sample volume, incubation temperature, and the exposure time (Fig. 9). The Bactiquant® assay have been applied in different RAS operating at different feed loading where Pedersen et al. (2017) demonstrated a high degree of correlation between bacterial activity and particulate surface area. Values ranging between 2.7×10^4 and 3.3×10^5 BQV/ml were found when operating RAS at feed loadings of 3.13 kg/m³ (Pedersen et al., 2012b; Paper II and III) while $0.61\text{--}1.3 \times 10^3$ BQV/ml was found during start-up of systems filled with tap water and without fish (**Paper I**).



Fig. 9: Diagram of bacterial activity measurement with Bactiquant®: 1) Water sample is filtered through a 0.22 µm filter where bacteria and organic matter is retained; 2) a specific amount of enzyme substrate is run through the same filter; 3) inside the filter, the substrate will react with the enzymes attached to the bacteria (30 min) and thereby, release the fluorophore; 4) Filters are flushed into a vial with liquid so the fluorophore are released into this liquid; 5) fluorescence of the liquid is measured and then the bactiquant value (BQV) is obtained by an equation provided by Mycometer AS.

The hydrogen peroxide (HP) degradation assay is a method introduced by Arvin and Pedersen (2015). Although HP is mainly known for its disinfection properties, it is also well known that microbial enzymes like catalase facilitate the degradation of HP in water (Hossetti and Frost, 1994). The method is based on adding a certain amount of HP to water samples, and then measure the HP degradation during time intervals over 60 minutes (Fig 10). The exponential reaction rate (k) is calculated from the exponential decay equation: $C_t = C_0 * e^{-kt}$, where C_t indicates the concentration of HP at time t , and C_0 represents the initial concentration, and k represents the constant rate of degradation in h^{-1} . The magnitude of the exponential reaction rate constant (k) value is directly correlated to the total bacterial activity in the water sample (**Paper II**).

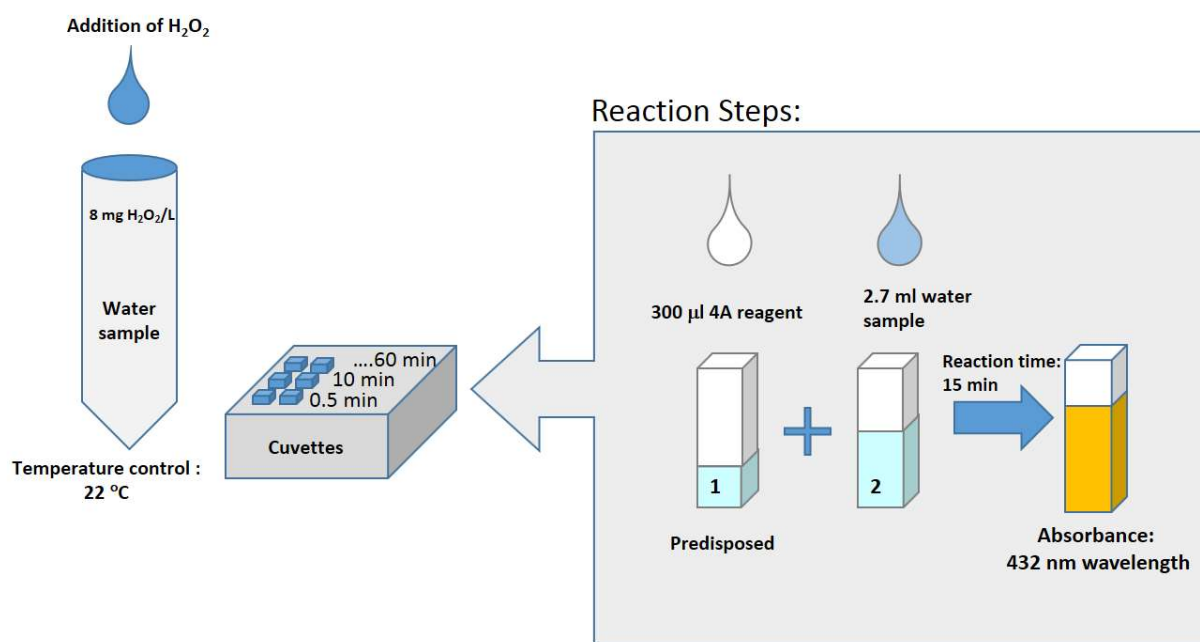


Fig. 10: Scheme of hydrogen peroxide (HP) degradation assay. The water sample is spiked at a concentration of HP under controlled temperature (22°C). The cuvettes are disposed and labelled beforehand with 300 μl of reagent 4A. Samples of 2.7 ml are taken before and 0.5 minutes after spike, followed by 15, 30, 45 and 60 minute's sampling. Samples reaction time is minimum 15 minutes to achieve change in color. Thereafter, samples are measured in a spectrophotometer at a 432 nm wavelength.

As an example, Fig. 11 from **Paper II**, shows degradation of HP within a time lap of 60 minutes after the addition of HP to water samples obtained from RAS operating at three different feed loadings. The water samples were measured for three consecutive weeks in all treatments. The degradation of HP was directly related to the microbial load within the systems. The higher the bacterial load the faster the HP degradation and considerable differences between treatments were observed during the 30 min of HP degradation.

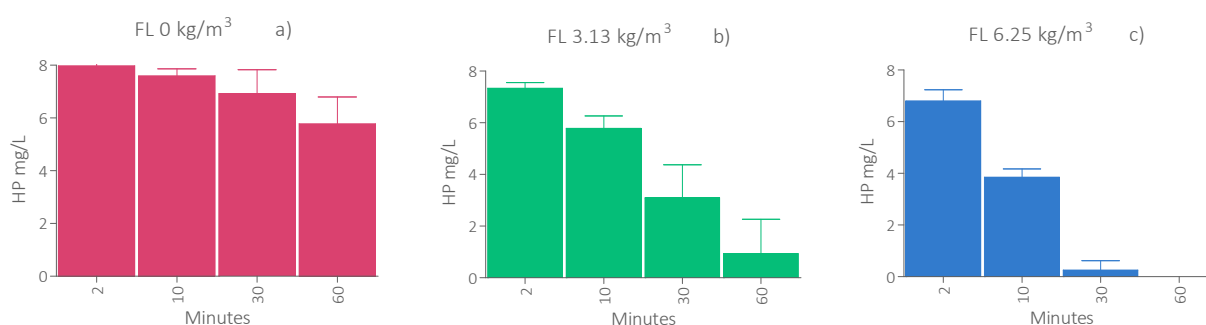


Fig. 11: Hydrogen peroxide concentration (mean \pm SD, $n=2$) measured through 60 minutes in water samples from three different feed loadings (FL): a) FL 0 kg/m^3 ; b) FL 3.13 kg/m^3 ; and c) 6.25 kg/m^3 (From: Paper II).

When looking at the rate constant (k) of HP degradation process (Fig. 12), there is also a clear indication on the level of microbial load. The higher the concentration of bacteria in the water, the higher the k .

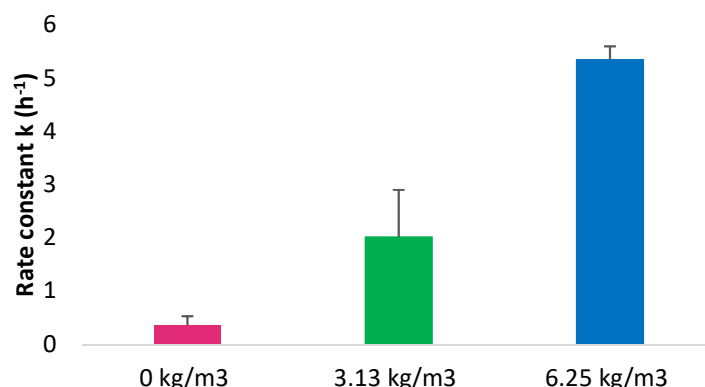


Fig. 12: Hydrogen peroxide degradation rate constant (k) according to different feed loadings (Data adapted from Paper II).

Hydrogen peroxide degradation has been shown to have a high correlation to the total number of particles within a 1-40 μm size-range (Fig. 13a) and good correlation to BQV has also been observed when comparing data from intensive RAS operating at 3.13 and 6.25 kg/m^3 (Fig 13b).

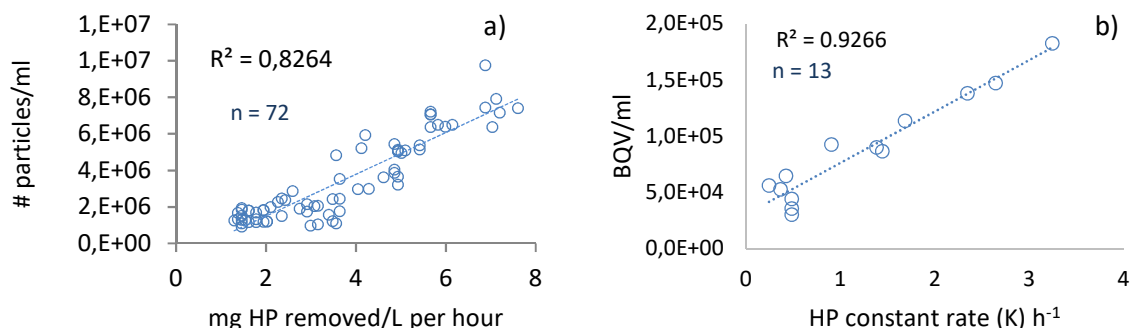


Fig. 13: Correlation between: a) HP degradation and number of particles ranging from 1-40 μm in diameter, and b) HP degradation rate and BQV (unpublished data).

The hydrogen peroxide degradation assay appear to have great potential for assessing microbial water quality within RAS water and can be adjusted for different water matrices. However, the method requires standardization in order to make it more applicable for use in commercial farms and for future comparison of data potentially obtained from other studies.

Another method of assessing bacterial activity in water is the Adenosine tri-phosphate (ATP) assay. This assay relies on measuring changes in ATP concentrations in water. Adenosine tri-phosphate is an energy-rich metabolic compound that is produced by all living organisms (Holm-Hansen and Booth, 1966). The ATP assay

is a fast method providing results within minutes (has also been tried as input parameter for on-line measurements) (Table 3) and has proven to be an applicable parameter for measuring active biomass in various aquatic environments (Holm-Hansen, 1966; Eydals and Pedersen, 2007; Siebel et al, 2008; Hammes et al., 2010). Recently, it has gained focus and can be used as a secondary assessment method to achieve a better picture of the operational efficiency of drinking water plants (van der Wielen and van der Kooij, 2010; Vital et al., 2012; Prest et al., 2016b). It has, however, not yet been used for assessing microbial biomass or activity in aquaculture water.

2.2.3 Bacterial communities

Beside bacterial abundance and activity, the type of bacteria and the composition of the microbial community can also be of major importance and these three variables could together influence fish welfare and performance (Blancheton et al., 2013).

Recently, sequencing techniques have contributed new information e.g. clarifying the general assumption whether ammonia- and nitrite-oxidizing species were identical in marine and freshwater environments (Tal et al., 2003). The techniques are presently developing fast and new applications might well appear in near future.. Many RAS studies have involved traditional microbiological techniques, targeted molecular methods as e.g. real-time polymerase chain reaction (PCR) or molecular fingerprinting analysis e.g. PCR-denaturing gradient gel electrophoresis (DGGE) (Sugita et al, 2005; Michaud et al., 2009; Wietz et al., 2009; Schreier et al., 2010; Tal et al., 2013; Kandel et al., 2014).

Deep sequencing is another emerging high-throughput technology for characterization of microbial communities in aquaculture systems. The technique is rapid and cost effective and provides an in-depth taxonomic characterization of the microbiota present, including unprecedented bacteria (Rud et al., 2017). The method allows for detection of thousands of bacteria in a sample and can monitor microbiota over time. Till now, the use of this technology has been limited to a few studies in RAS (Martins et al., 2013; Ruan et al., 2015; Rud et al., 2017) but further application combined with process information will likely provide new knowledge and understanding of the microbial communities.

3. Factors affecting bacterial dynamics in RAS

Vadstein et al. (1993) used the ecological theory of r/k-selection to describe the factors involved in the achievement of microbial stabilization in RAS water, focusing on the interaction of the carrying capacity with the r/k strategists. The carrying capacity is defined as the number of microbes that can be sustained within a system over time. The fast growing (opportunistic) bacteria are denominated as “r-strategist” (e.g. heterotrophs) whereas the “k-strategist” (specialized bacteria e.g. nitrifiers) are characterized by having a slower growth rate. The supply of organic matter within a RAS is typically the growth-limiting factor and determines carrying capacity. The r-strategists are opportunistic bacteria that typically favor unstable environments with little competition where they fast may occupy new available niches. K-strategist bacteria have a competitive advantage in substrate-limited environments operating close to the carrying capacity (Vadstein et al., 1993; Attramadal 2012a,b).

In RAS, the microbial environment is complex and several factors may influence the carrying capacity of a system, promoting imbalance in microbial water quality. The most relevant influential factors are depicted in Fig. 14 and will be addressed further in this section.

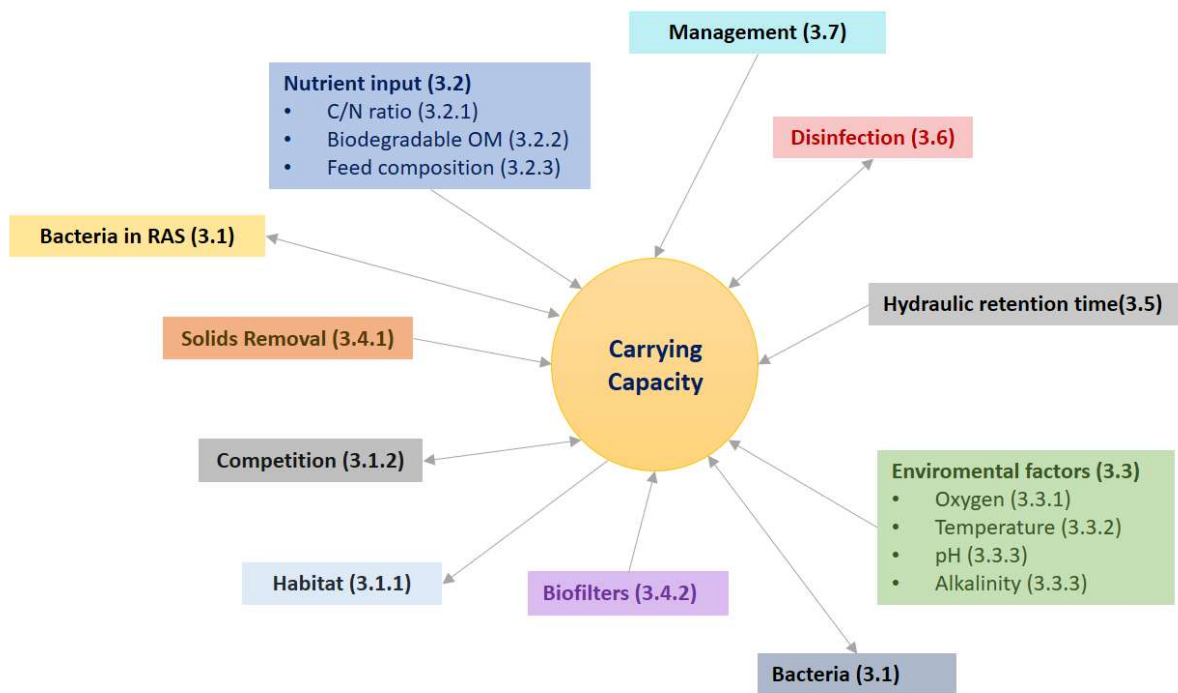


Fig. 14: Factors affecting the microbial carrying capacity in RAS water

3.1 Bacteria in RAS

The biofilter (after solid removal treatment) is generally the first treatment unit to receive water with high concentrations of organic and inorganic nutrients from the rearing units. Biofilter media provides a surface for bacteria to colonize (Polanco et al., 2000; Fernandes et al., 2017). In RAS, there are two main groups of bacteria: autotrophic and heterotrophic bacteria (Hagopian and Riley, 1998). The autotrophic bacteria are responsible for the nitrification process, where the ammonia-oxidizing bacteria (AOB) and ammonia oxidizing archaea convert ammonia into nitrite and the nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate (Hagopian and Riley, 1998; Schreier et al., 2010).

Nitrifying bacteria develop in clusters and attach to the support media (Schreier et al., 2010). The nitrification process is crucial because it keeps ammonia at sub-toxic levels (Timmons et al., 2009). This process may also affect water quality negatively due to bacterial oxygen consumption and reduction of the pH due to alkalinity consumption. Nitrification can be heavily impaired by changes in rearing conditions such as temperature, alkalinity, organic matter, dissolved oxygen, turbulence, salinity and ammonia concentrations (Nijhof and Bovendeur, 1990; Zhu and Chen, 2002; Chen et al., 2006; Timmons et al., 2009; Kinyage and Pedersen, 2016).

Due to the limited energy gain, autotrophs have a relatively lower growth rate than heterotrophic bacteria. If the conditions are suboptimal, autotrophs can risk being out-competed by heterotrophic bacteria (r-strategists), which have a faster growth-rate (0.1 vs 0.5 day^{-1} , Ebling et al., 2006).

Heterotrophic bacteria are the most abundant group within RAS (Leonard et al., 2000; Michaud et al., 2009; Michaud et al., 2014; Rud et al., 2017). In an ecological sense, most heterotrophic bacteria are considered to be “neutral” microbes as they somehow contribute to maintaining good microbial water quality by degrading the organic matter from where they obtain their energy (Bitton, 2011), and at the same time they occupy niches and may prevent the proliferation of harmful bacteria (Attramadal 2012a). The diagram in Fig. 15 depicts the contribution of both autotrophic and heterotrophic bacteria to RAS water chemistry, and the complexity of the different processes that affect chemical water quality.

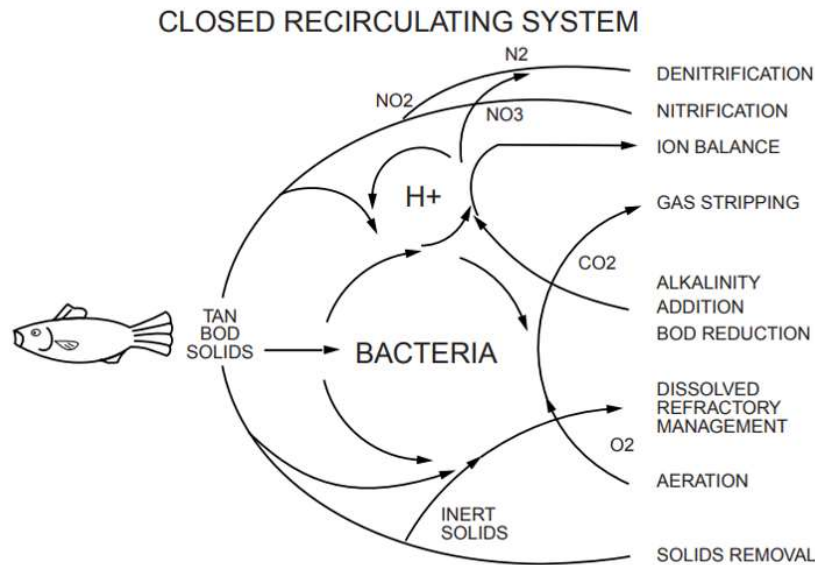


Fig. 15: Diagram of bacterial and chemical interaction associated to waste input within RAS (From: Masser et al., 1999).

Heterotrophic bacteria may also have some adverse effects such as: i) competition for oxygen consumption directly with the rearing organism or nitrifiers, ii) potential pathogens are found within their group (Blancheton et al., 2013; Rurangwa et al., 2015), iii) competition for space with autotrophic bacteria (Polanco et al., 2000; Nogueira et al., 2002). The equilibrium between nitrifiers and heterotrophic bacteria is dictated by the C/N ratio (discussed in section 2.1) and controlled by environmental conditions.

3.1.1 Habitats

Microbes in RAS has two major environments: i) surfaces such as biofilter, where bacteria metabolize waste compounds (NH_4 , NO_2 , organic matter); and ii) water, where bacteria interacts with fish, water and suspended micro-particles (Blancheton et al., 2013; Fernandes, 2015).

3.1.1.1 Surfaces

A well-known feature in bacteria is their tendency to form biofilms (McDaugald et al., 2012; Madigan et al., 2015). Bacteria forms biofilm for several reasons (Madigan et al., 2015):

- 1) It serves as a self-defense mechanism, providing protection from physicochemical disturbances and predation and thereby survival is increased.
- 2) The cells remains in a favorable niche where nutrients may be more abundant or are continually being replenished.
- 3) It facilitates cell-to-cell communication and there are more opportunities for nutrient and genetic exchange, increasing chances of survival.

3.1.1.2 Water

The water column is another important habitat for bacteria. In the water phase, bacteria can live attached to particles (Franco-Nava, 2004) or to the surface of higher order organisms (Liltved and Cribbs, 1999), or they can be free-living (same reference to planktonic, suspended cells or free-swimming cells). The dynamics and composition of the planktonic bacteria in RAS is not fully understood. In microbiology, planktonic growth is defined as a norm only for those bacteria adapted to live at very low nutrient concentrations (Mandigan et al., 2015).

In RAS, the removal of easy biodegradable organic matter is predominantly caused by the bacterial community inside the biofilter (Blancheton et al., 2013). The outlet water quality from the biofilter (that is recirculated) possesses different characteristics than the inlet water, due to the action of bacteria and the biofilter design (see section 3.4.2). Here, ammonia is transformed into nitrate, the bioavailable organic matter is metabolized to less degradable carbon compounds and the oxygen concentrations are reduced. Additional degradation of organic matter will occur after water passing through the degasser unit. All these factors affect the carrying capacity of the water. Despite the reduction in particulate and dissolved nutrients after the treatment units, micro-particles accumulate within rearing units and contribute to sustain microbial development (Blancheton and Canaguier, 1995; Wold et al., 2014). Bacteria attached to particles benefit from the protection from the particle (Hess-Erga et al., 2008) and they benefit from the nutrients available in/on the particle.

It is presumed that in the ocean, particle-attached bacteria transform the particulate matter into dissolved matter, and thus support the production rate of free-living bacteria (Cho and Azam, 1998). Bacteria attached to particles excrete enzymes that hydrolyze the particulate matter surface in order to obtain nutrients (Chróst, 1991; Smith et al., 1995; Eliosov and Argaman, 1995; Lee and Huang, 2013). These bacteria have significantly higher extracellular enzymatic activity per cell than the free-living bacteria (Karner and Herndl; Grossart et al., 2006). Smith et al. (1992) showed that they hydrolyze more organic matter than they take up. Based on this, it can be assumed that beside the limited availability of organic carbon for free-living bacteria in the recirculation loop within RAS, additional resources may be provided by the hydrolytic activity of bacteria attached to particles.

Moreover, bacteria in the ocean are characterized by being very small cells (less than 1 μm in diameter accordingly to Azam and Hodson (1977)) which is usually a characteristic of organisms living in a nutrient-poor environment. Small size is an adaptive feature for nutrient-limited microorganisms due to the reduced energy requirements for cellular maintenance (Madigan et al., 2015).

There are very few data on the number of planktonic bacteria in the water phase of large commercial RAS under normal conditions. Experiments with marine larvae reared in RAS have reported values of 10^6 to 10^7 cells/ml (van der Meeren et al., 2011; Attramadal et al., 2014; Wold et al., 2014). Inlet seawater contained 10^6 cell/ml (van der Meeren et al., 2011; Mandigan et al., 2015) and tap water 10^3 to 10^6 cell/ml (Prest et al., 2016a).

Large commercial RAS have tanks of 1000 m^3 in water volume containing around 50 tonnes of fish and each RAS facility will have a number of these tanks connected to a common water treatment unit (Fig. 16). Here,

bacteria have an enormous space to live as free-living bacteria or attached to suspended particles, sharing at the same time space with the rearing species of high monetary value. The surface area provided just by the particles present in the water column is of special concern, considering rearing volumes in innovative RAS shown in Fig. 16. In a water sample from a commercial RAS system, the number of particles can be around 10^6 particles per ml ranging from 1 to 30 μm and the calculated particulate surface area amounts to $17 \text{ m}^2/\text{m}^3$ water. This illustrates the huge surface area per cubic meter available for bacteria to grow on in a system as shown in Fig. 9. The advantage is that RAS operation with an even supply of organic matter exerts a stronger selection pressure on microbes, and thereby a more stable microbial water quality can be achieved (Attramadal et al., 2012a). Presumably, this contributes to establish a higher diversity and beneficial bacterial communities in the water (Verner-Jeffrey et al., 2003).

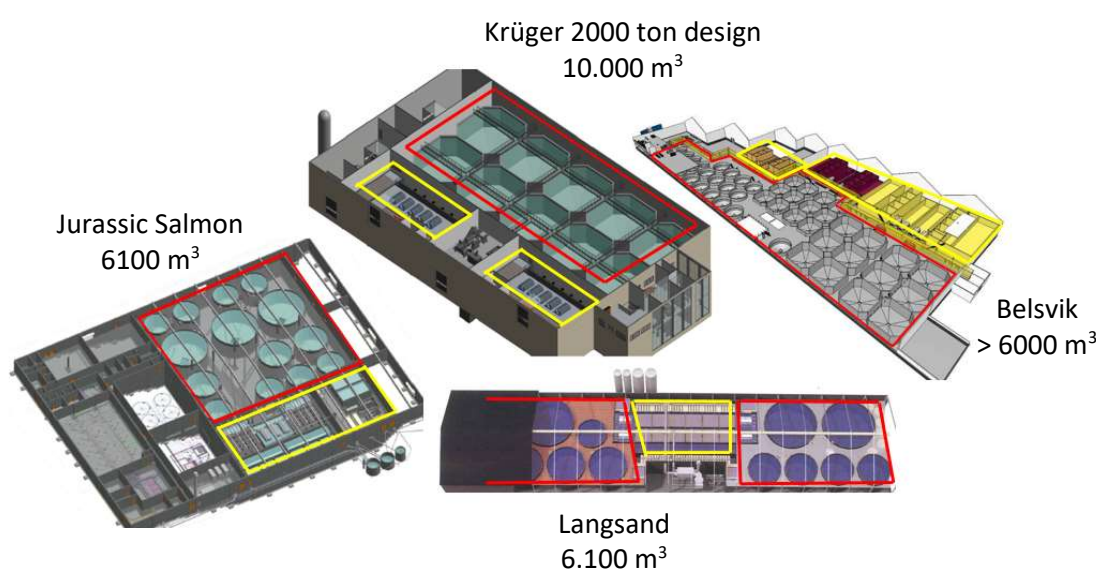


Fig. 16: Recirculating aquaculture systems designed and constructed recently and their volumetric capacity in terms of rearing water. Red areas are the rearing systems and yellow areas are the treatment units (Source: Billund Aquaculture AS, Lerøy Midt AS, Krüger Kaldnes AS).

3.1.2 Predation

In recirculating aquaculture systems as in every aquatic environment, has its own ecosystem shaped and defined by its specific abiotic and biotic factors. Within the RAS ecosystem, organisms and operational conditions are inter-related by the transfer of nutrients and energy through a food web. Moreover, in this food web there are habitats, niches, and relationships (symbiotic and predator-prey). Aquatic bacteria are better at competing for soluble substrates than protozoa, but the bacteria serve as particulate substrate (prey) for protozoan and metazoan predators (Gerardi, 2006). Predation generates selective pressure on microbial abundance and normally their presence is the consequence of in the high bacterial abundance (Prest et al., 2016a). It is also known that protozoa and metazoa selectively feed on larger bacteria (Boenigk et al., 2004). Within the planktonic bacterial community, particle-associated bacteria are a relatively easier

target for filter feeders than free-living bacteria. Bacteria attached to particles can be directly consumed by planktonic metazoans, bypassing consumption by protozoan and thus shortening the microbial loop (Gonsalves et al., 2017). Simple observation of the existence of different types of organisms within RAS, might give indications on system/treatments performance.

3.1.3 Bacterial dynamic

The assessment of microbial water quality in this thesis, was mostly performed in RAS operated under constant conditions (in terms of amount of feed and water exchange) for a prolonged period of time. Microbial growth in RAS operated in these conditions, could be related to the growth curve obtained in a continuous reactor as shown in Fig. 17.

The bacterial growth curve can then be divided into the following phases (Gerardi et al., 2006):

- Lag phase: The lag phase of growth occurs during the start-up and also in a recovery state. The length of the lag phase is determined by the conditions of the new environment and the species of bacteria present. Here, bacteria do not reproduce but they are synthesizing enzymes to degrade substrate in the new environment.
- Log phase: The next phase is called log phase, which is related to the bacterial characteristic to grow at a logarithmic rate. It can be divided into three phase: i) uptake of substrate, ii) cell synthesis and rapid growth, iii) cell synthesis and declining growth. During substrate uptake, bacteria duplicate and biomass increases. Due to the improved enzymatic mechanism during the lag phase, bacteria can degrade substrate and thus reproduce. At the cell synthesis and growth decline step, the growth rate decreases because bacteria are limited by the available/assimilable substrate and/or the space may become limited. At this point, the population of bacteria is reaching the carrying capacity or maximum number of organism that a system can support.
- Endogenous phase: Finally, the endogenous phase of growth (also called stationary or equilibrium) is considered when bacteria have reached the carrying capacity of the system. Here, is where it is important to differentiate RAS under experimental conditions (as the reactors) and RAS under normal rearing operations. Under normal operational conditions of a RAS, the carrying capacity is dictated by the feed supply. Feed might be increased to obtain a desired growth and, theoretically, the carrying capacity should thus expand with increased feeding. Further evaluation on this matter is required in order elucidate bacterial dynamics in relation to normal operational conditions.

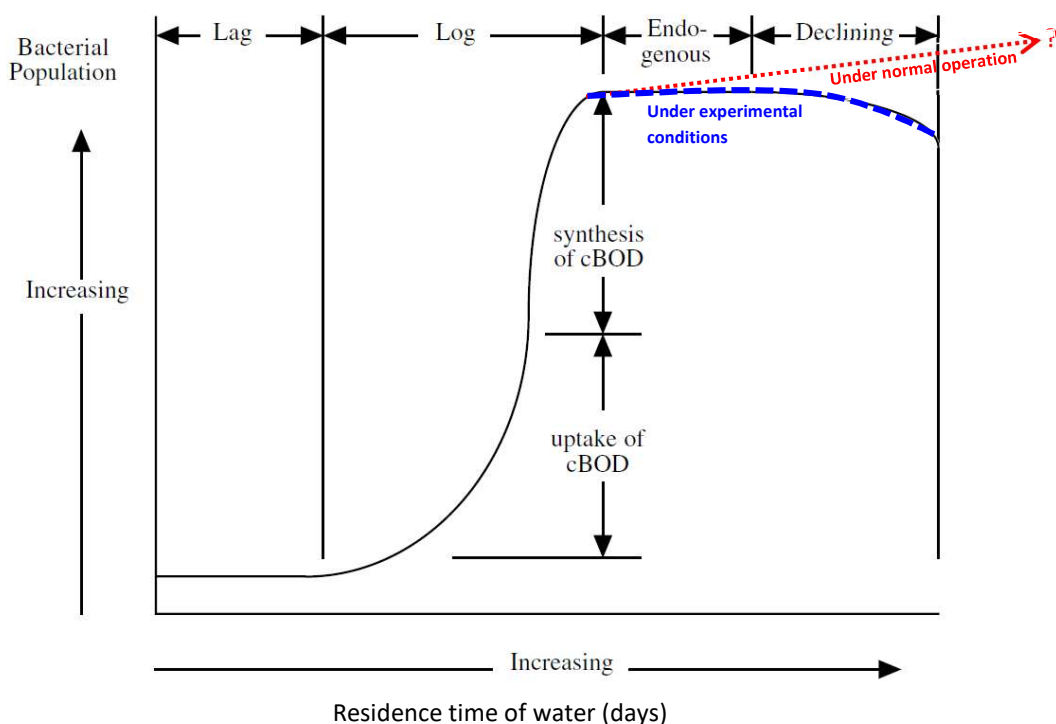


Fig. 17: Bacterial growth-curve in a continuous culture/reactor to explain bacterial growth in RAS under normal and experimental conditions (Adapted from Gerardi, 2006).

The first microbial fingerprint in every RAS occurs during the start-up of a system where system water and biofilter media become colonized by bacteria (lag-phase). Bacteria can be introduced from inlet water, from fish intestinal microbiota (Sugita et al., 2005; Giatsis et al., 2015) and external sources such as feed, air-borne, equipment, staff, etc. (Sharrer et al., 2005; Blancheton et al., 2013). During the start-up, organic matter accumulates and bacteria start to increase in abundance and activity (Fig. 18) (**Paper I**). The start-up of RAS is a vulnerable phase where bacteria are exposed to selective processes due to the addition of substrate. If the supply of organic matter is not well controlled, the r-strategists will prevail and be the first to colonize the free niches and thereby delay the start-up performance of the biofilter. It takes days for RAS to reach the steady state within a biofilter (Timmons et al., 2009). Under operational conditions, the steady state of a system is normally represented by constant low concentration of TAN and nitrite, and the accumulation of nitrate (Colt et al., 2006).

Systems operating under constant feed input and water exchange (Fig. 18), indicates that when monitoring the different fractions of organic matter in the water phase (particulate and dissolved COD; $COD_{TOT} = COD_{DISS} - COD_{PART}$), initially both accumulates over time (Fig. 18). Moreover, the dissolved fraction stops decreasing after approximately 60 days whereas the particulate fraction continues to increase. The free-living bacteria increase within the first thirty days then to stabilize between $0.8 - 3 \times 10^4$ cells/ μ l. Furthermore, bacterial activity - which is related to particulate surface area - is increasing consistently with the particulate COD (Fig. 18). The presence of free-living bacteria and particle-associated bacteria in RAS water is regulated by the interaction of the biofilm in biofilter and bulk water (Leonard, et al., 2000, Paper III) but within the water

phase, another interaction between the free-living bacteria and particle-associated bacteria seems to be important and needs further attention.

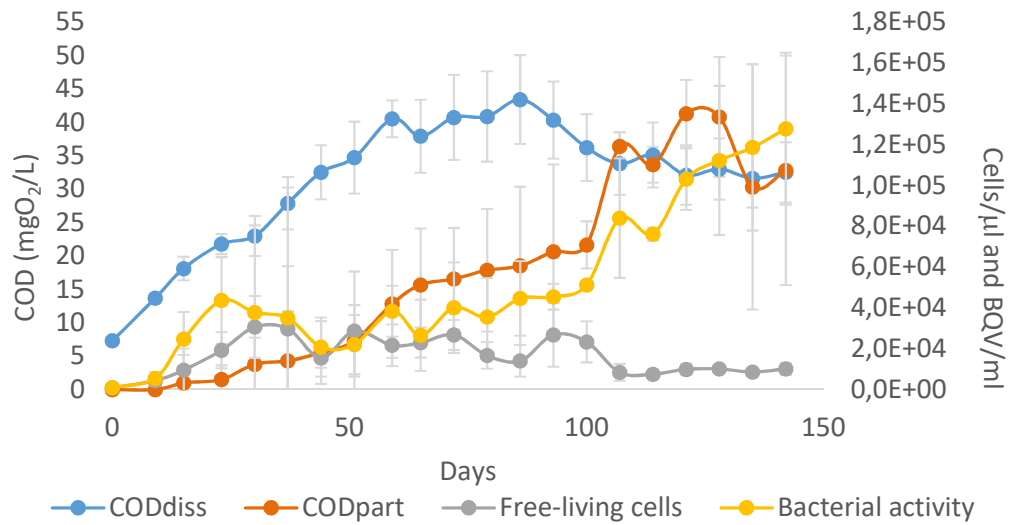


Fig. 18: Concentration of particulate COD and dissolved COD_{0.2μm} plotted with bacterial activity and abundance from a RAS operating under constant conditions (3.13 kg/m³) within 140 days (Data adapted from Paper I and II).

3.2 Organic and inorganic nutrients

Digested and any undigested feed is the main input of organic matter into the water in RAS. Any uneaten feed and fish excretion (waste) compose the main source of resources for bacteria to grow in RAS water (Fig. 19). Fine particulate organic matter that is not removed during mechanical filtration will be further degraded until eventually contributing to the dissolved fraction of organic matter. The dissolved fraction can be divided into two sub-fractions, inorganic nutrients and organic nutrients (Fig. 19). The inorganic nutrients are compounds such as nitrogen, phosphorus or trace elements and organic nutrients include the organic carbon in all kinds of forms (Prest et al., 2016a). The turnover of the nitrogenous compounds is taken care of by nitrifying bacteria, which obtain their energy by oxidizing nitrogen and consuming inorganic carbon (CO_2) for cellular synthesis (Gerardi, 2006).

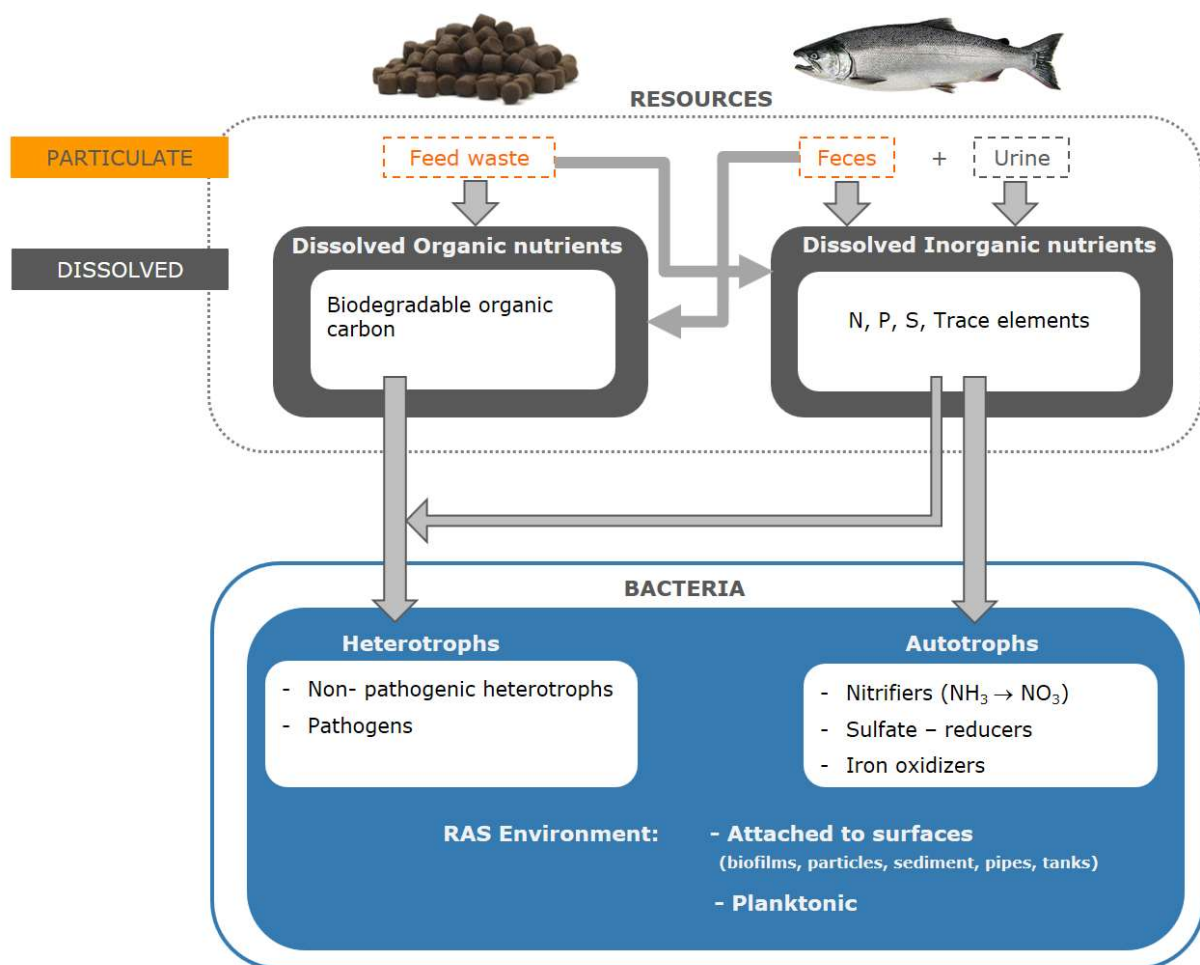


Fig. 19: Characterization and dynamics of the resources available for different types of bacteria in RAS (Adapted from Prest et al., 2016a).

The ratio of the elemental composition of bacteria of carbon, nitrogen and phosphorus (C:N:P) is 100:10:1 (van der Kooij and Hijnen, 1982) and carbon is most often the growth-limiting compound in RAS water (Leonard et al., 2002). According to the literature, a dissolved carbon concentration of 1 µg C/L is enough to promote the growth $10^3 - 10^5$ cells/ml (Vital et al 2012; Prest et al., 2016a). Dalsgaard and Pedersen (2011) have shown that 81 mg of COD per g feed is recovered in the water phase after feeding. Taking into consideration the ratio of carbon to oxygen: 12 g mol of carbon to 32 g mol oxygen (Ebeling et al., 2006), the amount of carbon in the water would be 30.37 mg carbon per g feed. Accordingly, there is enough organic carbon to sustain a limited growth of heterotrophic bacteria in RAS water under operational conditions where nitrogen (N) and phosphorus (P) ranges around ~ 130 mg N/L and ~ 3 mg P/L (Pers. obs).

Organic matter compromise a broad spectrum of different organic carbon compounds ranging from simple organics to more complex polymeric substances as e.g. humic compounds (Prest et al., 2016a; Hambly et al., 2015). Therefore, only a fraction of the biodegradable organic matter can be readily utilized for bacterial growth. In drinking water, it is estimated that 0.1 and 30% of the total DOC is assimilable by bacteria (reviewed in Prest et al., 2016a). The range of available assimilable carbon for RAS water is not clear.

3.2.1 C/N ratio

Several studies have focused on the carbon/inorganic nitrogen ratio (C/N) as a factor that affects biofilter performance. At a high C/N ratio, heterotrophic bacteria out-compete nitrifiers for available oxygen and space (Avnimelech, 1999, Zhu and Chen, 2001, Nogueira et al., 2002, Michaud et al., 2006, Michaud et al., 2014), potentially causing a decrease in the nitrification rate (Fig. 20). However, there are other hydraulic and physical interactions that could cause over/underestimation of the C/N ratio.

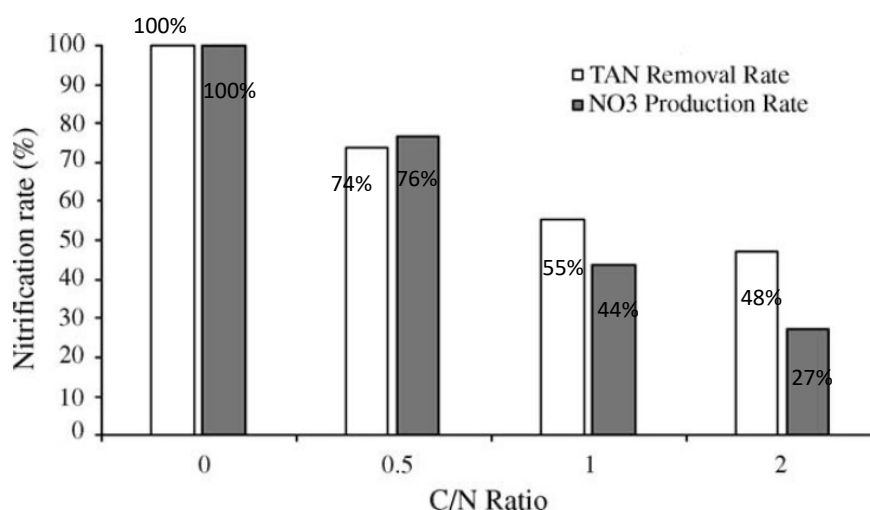


Fig. 20: The effect of increasing carbon to nitrogen ratio on nitrification rate (From: Michaud et al., 2006)

Higher C/N ratio are still possible to handle if two main interactions are considered: i) the dissolved oxygen should be kept close to saturation (Zhu and Chen, 2003); ii) rapid water fluxes will decrease biofilm and diffusive boundary layer thus substrate penetration can be maximized and thereby, nitrification will be sustained by maintaining a high dissolved oxygen concentration in the bulk phase (De Beer et al., 1996; Prehn et al., 2012).

By constantly increasing the feeding load, the concentration of carbon and nitrogen increase in the water without changing the C/N ratio. Thus, bacterial load should increase (**Paper II**). However, the C/N ratio might significantly increase in case of failure solid removal system, biofilter backwash or in case of feed spill (**Paper III**), which are the major concern of possible addition of carbon to RAS water showed in Table 4. Considering that BOD assess only the easily biodegradable part of organic carbon compounds, in large commercial scale RAS the organic matter might be fully degraded due to the high residence time water have within the system (hydraulic retention time can be up to 7 weeks).

Table 4: Carbon content in different organic matter forms and fractions.

Form of organic matter	COD^a mg O₂/ g feed	Total carbon^{b,d} mg C/ g feed	BOD₅^a mg O₂/ g feed	Biodegradable carbon^{b,e} mg C/ g feed
Suspended ^c	81	30	41	15
Feaces	198	74	32	12
Feed	1300	488	-	-

^a Values obtained from Dalsgaard and Pedersen (2011).

^b Calculated accordingly to ratio of carbon to oxygen (0.375 g C/g O₂) (Ebeling et al., 2006).

^c Remains in the water.

^d Calculated from COD

^e Calculated from BOD₅

3.2.2 Biodegradability index

The biodegradability index is defined as the ratio between BOD and COD (BOD/COD) and can be used to characterize the biodegradability of the organic matter in water. Dalsgaard and Pedersen (2011) characterized the waste production from trout and described the characteristics of removed solids and suspended/dissolved organic matter remaining in the water. They elucidated that there was a higher biodegradable index within the organic matter retained in the water phase than the ones in the feaces (Table 4).

In wastewater, this index is widely used to assess the biodegradability of the domestic waters and thereby decide if it is suitable for biological treatment. The following classification is suggested according to Srinivas (2008): if BOD/COD is > 0.6 the organic matter is easy biodegradable, if BOD/COD is between 0.3 and 0.6, the organic matter is average biodegradable, and if BOD/COD is < 0.3 the organic matter is not easily biodegradable. In RAS systems operating under constant conditions for a long period (≥ 3 months) biodegradability indexes ranging from 0.08 to 0.1 have been shown (Fernandes et al., 2015; **Paper I and II**).

This suggests that there is a low fraction of easily biodegradable organic matter available in the water of RAS thus generally operating close to the carrying capacity (endogenous phase, Fig. 17).

3.2.3 Feed composition

Traditional aquaculture feed diets relies on fishmeal as the traditional protein source (Naylor et al., 2000). Due to economic and pressure on wild fish stock, alternative feed diets formulation without fishmeal have become the potential solution (Heikkinen et al., 2006).

Studies have suggested that alternative and traditional diets may cause intestinal disorders (Baeverfjord and Krogdahl, 1996; Green et al., 2013, Ingerslev et al., 2014) and thus potentially affect water quality in farms.

Concerns on how alternative ingredients may affect microbiota within RAS water have also been addressed. Schmidt et al., (2016) showed that fishmeal-free diets had an influence on the microbial community in salmon intestines. However, they did not find that these alternative diets influenced the taxa in RAS water of systems that were acclimatized two months previous to the diet trial.

3.3 Environmental factors

The control of environmental conditions in RAS is an advantage. Control of pH, alkalinity, oxygen and temperature are some of the variables that can be controlled. Changes in these variables can affect microbial water quality in different ways and some of them are addressed in this section.

3.3.1 Oxygen

Dissolved oxygen in RAS is a critical and important parameter due to reared species requirements (Timmons et al., 2009) and the requirements of bacteria in the biofilter. Due to this, oxygen is a tightly controlled parameter in RAS and maintained according to the desired levels. Biofilter units often have an extra supply of oxygen to maintain proper function. It has been suggested that the nitrification processes may be affected under concentration of 2 mg/L dissolved oxygen (Timmons et al., 2009). However, for operating a biofilter with such low oxygen concentration, factors such as good flow within the biofilter unit, biofilm structural condition, concentration of TAN and nitrite and the type of design of the biofilter, have to be properly dimensioned.

3.3.2 Temperature

Temperature is an important parameter for the reared species but also an important factor affecting bacterial growth, kinetics and competition processes (Prest et al., 2016a). Traditionally, temperature is controlled within commercial RAS.

Temperature exerts two significant effects on bacterial populations: i) it affects the rate of diffusion of organic and inorganic nutrients into bacterial cells and ii) it affects the rate of enzymatic activity. With increasing temperatures, the rate of diffusion of substrate into bacterial cells increases, and the rate of enzymatic activity increase (Gerardi, 2006). Changes in temperature trigger heterotrophic activity which increases degradation of organic matter and the BOD:COD ratio is affected. Nitrification rate is a sensitive process depending on temperature, the higher the temperature the higher the turnover rate (Fig. 21).

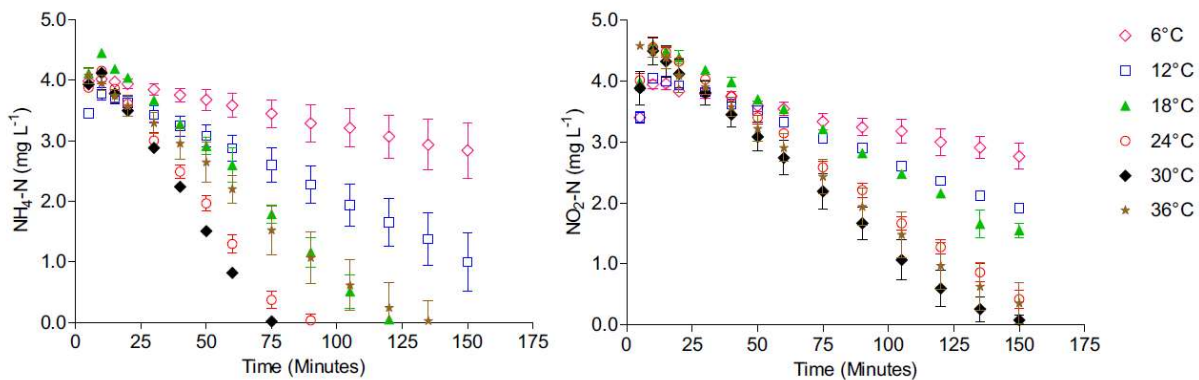


Fig. 21: Temperature effect on nitrification rate on moving bed bio-elements from a matured freshwater RAS (Kinyage and Pedersen, 2016).

3.3.3 pH and alkalinity

RAS water quality requirements have to reflect the reared species requirements. The relative proportion of ammonia (NH_3) and ammonium (NH_4^+) change at different pH values (Allison and Prosser, 1993). At higher pH, the NH_3 concentration increases in the water. As NH_3 is toxic at very low concentrations, controlling pH at levels is crucial in RAS water quality management (Timmons et al., 2009; Colt, 2006). A neutral pH is required in order to match the optimal requirements for both nitrification and the health/performance of most commercially reared species (Chen et al., 2006).

A decrease of pH below 6.8 is a matter of concern in systems operating with saltwater due to sulfides, which can trigger autotrophs to produce H_2S (hydrogen sulfide) (Gerardi, 2006). Hydrogen sulfide is toxic for fish at nearly undetectable concentrations.

3.4 Treatment components in RAS

Accumulation of solids in RAS can lead to a decline in physicochemical and microbial water quality and can affect the health of the reared organisms. Treatment components in RAS and system design parameters may have significant effects on microbial water quality. In this section, provides general information on how solid removal and biofilter units may affect microbial water quality within RAS.

3.4.1 Solids removal

Removal of solids is of high priority within RAS (Cripps and Bergheim, 2000). Large particles can be removed from RAS by the use of different technologies such as drumfilters, sedimentation ponds/tanks, swirl separators, sand filter *etc.* (Timmons et al., 2009). The selected technology for solid removal will define the size of solids accumulating within the RAS. The most common mechanical filtration used is drumfilters (Fernandes et al, 2015). Smaller particles tend to remain within the system, accumulating over time (Chen et al., 1993; Fernandes et al., 2014, 2015; Becke et al., 2017b) and undergo further disintegration due to shear forces from e.g. pumps, water falls, biological degradation (McMillan et al., 2003; Sindilariu et al., 2009, Wold et al., 2014).

Suspended particles in intensive RAS originate from larger particles coming from feed waste and from faeces (Dalsgaard and Pedersen, 2011; Pedersen et al., 2017). Becke et al. (in prep.) found a direct correlation between total suspended solids from drumfilter backwash and bacterial activity (Fig. 22) which suggest that particles are highly colonized by bacteria potentially, preceding colonization inside fish digestive tract has occurred). The particle residence time in systems increases microbial colonization on active or inactive particles (Brinker and Rösch; Pedersen et al., 2017).

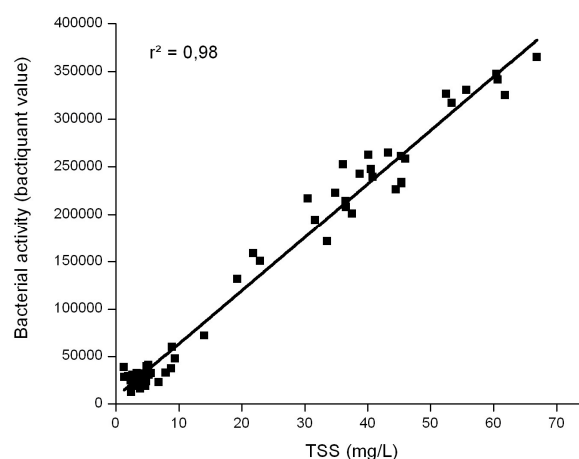


Fig. 22: Correlation between total suspended solids from drumfilter backwash and bacterial activity measured with Bactiquant® (Becke et al., in prep.).

In RAS, approximately 95% of the micro particles accumulating within the system is $< 20 \mu\text{m}$ (Chen et al., 1993; Fernandes et al., 2015). In Paper I and II, it was found that $> 95\%$ of these particles ranged between 1 to $3 \mu\text{m}$. A subsequent study when using flow cytometry (Paper III) showed that the concentration of free-living bacteria below $1 \mu\text{m}$ was 10^6 cell/ml .

Optional treatments can be applied for removing fine particles and thereby improve water quality conditions. Membrane filtration technology has been used for larvae RAS systems, where water is filtered from 0.1 to $10 \mu\text{m}$ or ultrafiltrated from 0.001 to $0.1 \mu\text{m}$. Thus, membrane filtration has the ability to remove micro particles from water and thereby prevent the accumulation of fine ($< 35 \mu\text{m}$) and colloidal particles ($< 0.1 \mu\text{m}$) in RAS (Wold et al., 2014). Newly hatched marine fish larvae are sensitive to infections caused by opportunistic bacteria and this is due to they possess an immature specific immune system (Vadstein, 1997; Skjermo and Vadstein, 1999; Sandlund et al., 2010). This kind of technology has been shown to have a positive effect on the performance and survival of larvae (Wold et al., 2014). The application of this technology in larger systems would probably not be economical feasible due previous filtration steps would be required because of the amount of organic load larger systems operate at.

Foam fractionators (protein skimmers) are another removal treatment where particles $< 20 \mu\text{m}$ can be removed. These devices remove particles at higher efficiency in seawater due to the bubble size/surface tension condition. Foam fractionation has shown improved particle removal when applied together with ozone, which also destroys and removes bacteria (Park et al., 2011).

In salmon farms, foam fractionation and ozone are used to increase water clarity and thereby increasing visibility of feed pellets in saltwater (pers. obs.).

3.4.2 Biofilter

Biofilter bacterial communities have a direct and indirect effect on bacterial abundance in the water phase (**Paper III**). Some studies have shown that the abundance of free-living bacteria in RAS rearing water can be correlated to the abundance of attached bacteria to biofilter media (Leonard et al., 2000; Michaud et al., 2006). Biofilm dispersion has become a well-recognized natural phenomenon linked to the terminal stage of biofilm development and is process for regulating biofilm structure (Davies, 2011; McDougald et al, 2012).

There are two ways of escape from biofilms:

- i) Release of individual cells or groups from a bulk liquid interface or from the outer layer of a biofilm. Cells or clusters of cells may transition directly from the biofilm into the bulk liquid, action that is defined as “detachment”;
- ii) cells escape from the interiors of a biofilm where they are not in direct contact with a bulk liquid interface. Here, bacteria must work their way through zones of overlying or adjacent cells to exit the biofilm. This type of escape is referred to as “dispersion”.

Detachment of cells from a biofilm can occur through four different mechanism i.e. abrasion, grazing, erosion, and sloughing (Fig. 23). Abrasion is the release of cells from the biofilm due to collision of large particles coming from the bulk liquid. This type of physical mutilation is very typical within fluidized bed biofilter and sand filters, and is likely the principle factor preventing biofilm accumulation. Grazing is the removal of biofilm caused by feeding activity of higher trophic level organisms. Erosion is the constant loss of small segments of biofilm due to fluid shear force in the biofilter. Erosion can also be caused by the shearing effect of gas or air bubbles transported through the layers of the biofilm or coming from the bulk liquid. Finally, sloughing is the result of the removal of larger segments of the biofilm due to e.g. fluid shear. In some occasion, the entire biofilm detach and enter the bulk liquid (Davies, 2011).

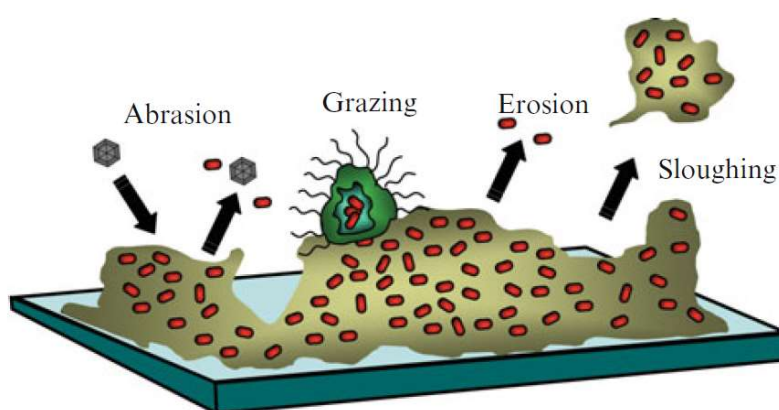


Fig. 23: Four mechanisms that influence biofilm detachment: abrasion, grazing, erosion and sloughing (From: Davies, 2011).

As detachment comprises release of cells due to physical forces, dispersion is the release of live bacteria from a biofilm as a result from physiological responses to internal or external stimuli. Dispersion of bacterial cells

from biofilm is a seeding process that leads to translocation of bacteria to colonize new habitats. Dispersion can also be a response mechanism under unfavorable conditions *i.e.* depletion of resources and competition (starvation), aging/maturation of biofilm, and stress due to grazing, presence of bacteriophages and/or toxicity (Davies and Marques, 2009; Davies, 2011; McDougald et al., 2012).

The main advantages and consequence of this mode of action, is availability to colonize a greater range of habitat types. Secondly, dispersion avoids inbreeding, thus prevailing fitness and generation or maintenance of genetic variation (McDougald et al., 2012).

Dispersion is an essential mechanism within the life cycle of a biofilm (Fig. 24) where dispersion of cells can be characterized as active or passive. The active dispersal of cells is the physiological response of bacteria in the biofilm to external/internal stimulus whereas passive dispersal due to detachment mechanism. Both types of dispersal response results in the release of free-living bacteria that may undergo attachment and form further micro colonies (Davies, 2011; McDougald et al., 2012)

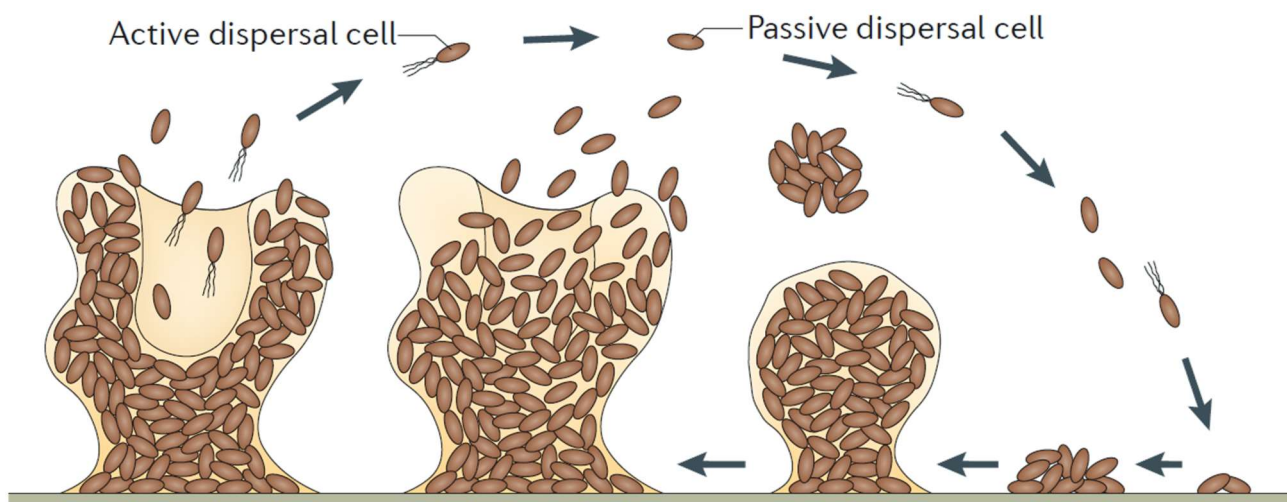


Fig. 24: Biofilm life cycle considering active and passive dispersal (From: McDougald et al., 2012)

During the design of a RAS, the biofilter is dimensioned to support the maximum capacity of feed per day within the production cycle. In this sense, it is expected that space is not a problem for bacteria to grow/colonize, especially when the supply of feed is done under controlled operational conditions.

There are three types biofilter commonly used within RAS, these are: fixed bed, moving bed and trickling filter. These types of biofilters may affect microbial water quality in different ways.

Fixed bed biofilter

Fixed bed biofilters generally contribute to the removal of particles by entrapping them but particles can also be released due to factors such as aging of the biofilm. Fixed bed biofilters are also acknowledged for removing biodegradable organic matter (Fernandes et al., 2017).

Moving bed biofilters

Moving bed biomedias are small plastic elements suspended in the reactor by aeration (Åhl et al., 2006). The aeration is the mode of motion that creates turbulence, providing the movement and mixture of the biomedias inside the reactor. The turbulence in the reactor contributes to the transport of the substrate to the biofilm and serves as a self-cleaning mechanism where shear forces act on the outer layer of the biofilm maintaining a low thickness (Rusten et al., 2006). For this reason, moving bed biofilters produce micro particles (Fernandes et al., 2017) which may sustain bacterial growth further in the recirculation loop.

Trickling filter

Trickling filters consist of a fixed media through which pre-settled or micro screen filtered rearing water trickles down across the height of the trickling filter. As water trickles down, it is oxygenated while the carbon dioxide is degassed. The trickling action contributes to particle disintegration contained in the rearing water or from biofilm shedding (Eding et al., 2006) which could also contribute to further microbial growth in RAS.

3.5 Hydraulic retention time

Hydraulic retention time within RAS is the time a water volume resides within a treatment or rearing unit/tank. It can also express the average retention time in a RAS based on the overall volume of the system in relation to the make-up water. This is the main advantage of RAS in terms of microbial maturation as bacteria are kept inside the system. Furthermore, it is presumed that the longer time water resides inside the system, the easier it is to preserve microbial maturation (Attramadal et al., 2014).

Studies in relation to drinking water have shown that the higher the residence time is within the system, the higher is the bacterial abundance observed (Reviewed by Prest et al., 2016a). Other studies have shown high microbial diversity in RAS when compared to a flow through systems due to the HRT of RAS systems (Attramadal et al., 2012a,b). The effect of HRT on microbial water quality within RAS is not clear and it is often dependent on other factors as e.g. feed loading, the efficiency of treatment units and disinfection procedures.

3.6 Disinfection

In most RAS, permanent or periodic water disinfection methods are applied as part of the management of systems to control or eliminate pathogenic organisms (Pedersen et al., 2012a) and to improve water quality (Goncalves & Gagnon, 2011).

Recirculating aquaculture systems have a defined carrying capacity and HRT that provides a stable microbial succession over time. This provides the ideal conditions for the establishment of *k*-selected species and thereby biological stabilization is achieved easier. This is, however, challenged by disinfection practices, which may intermittently destabilize microbial water quality (Attramadal et al., 2012b).

Disinfection procedures in RAS are often based on oxidative agents as e.g. ozonation, peracetic acid, hydrogen peroxide addition or other types of disinfection like UV irradiation, chloramine – T and formalin (Sharrer & Summerfelt, 2007; Pedersen et al., 2010; Attramadal et al., 2012b; Pedersen and Pedersen, 2012; Pedersen et al., 2013). These disinfectants can create changes in microbial water quality due to combined effects of i) increased nutrient availability and ii) elimination and/or inactivation of bacterial cells, thus creating a new niche for bacterial growth (regrowth potential for r-strategists) (Hess-Erga et al., 2010; Attramadal et al., 2012b; Prest et al, 2016a).

Disinfection can be applied in RAS at different points within the system. Traditionally, the inlet water is disinfected (point 1 in Fig. 25) by the application of UV irradiation in order to prevent the entrance of potential pathogens from the external water source (Timmons et al., 2009). Within the recirculation loop, disinfection can be applied before the biofilter by the use of UV and/or ozonation (Sharrer & Summerfelt, 2007; Attramadal et al., 2012b). Placing disinfection before biofilters allows the bacterial community inside the biofilter to cope with the available active agent (point 2, Fig. 25). Disinfection before the rearing tanks (point 3, Fig. 25) has been shown to potentially select for opportunistic bacteria in the rearing units (Øien, 2014). Finally, disinfection directly in the rearing tanks is done by the addition of therapeutic agents (e.g. peracetic acid, hydrogen peroxide, formalin and chloramine-T) directly into the rearing units for treating the reared species directly (point 4, Fig.25) (Pedersen et al., 2010; Pedersen & Pedersen, 2012; Pedersen et al., 2013).

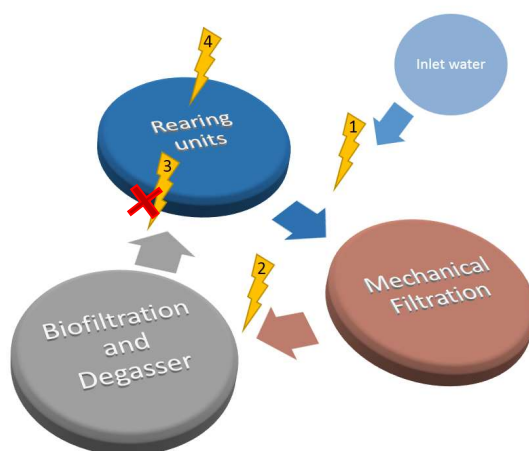


Fig. 25: Disinfection contribution when applied in the different locations: 1) after inlet water, 2) before biofilter, 3) before rearing units/tanks, and 4) direct application in rearing tank water.

The application of disinfection before the rearing units has shown to be unsuccessful. Øien (2014) applied UV disinfection before rearing units on a lobster larvae RAS, which resulted in lowest survival in larvae compared to a RAS without disinfection and a flow-through system (Fig. 26,a). In addition, the microbial community assessment showed that the RAS without disinfection had a higher diversity compared to the other two systems. It was suggested that the disinfection may have affected the recolonization of the

microbiota in water and thereby the microbial community. Another study by Attramadal et al. (2012b), showed that the recolonization and development of microbial community in a marine larvae RAS was affected when applying UV and ozonation disinfection (both disinfection units placed before biofilter) (Fig. 26, b). The changes in bacterial abundance between the disinfected treatment systems were explained to differences in the disinfection efficiencies. This suggests that the difference between microbial abundance and communities was due to the imbalance of the resulting carrying capacity of the treated waters and the amount of bacteria competing for substrate.

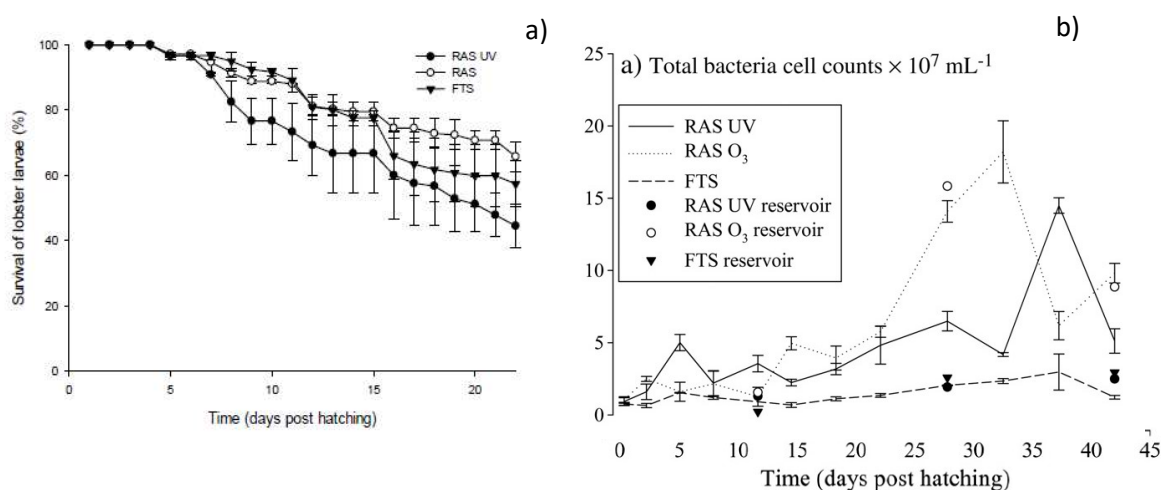


Fig. 26: a) Effect on lobster larvae survival reared in RAS with UV disinfection applied before rearing units (Øien, 2014); b) effect of disinfection treatments on microbial abundance from marine larvae RAS (Attramadal et al., 2012b).

The recommended dosage concentrations of therapeutic chemicals have to take biofilter performance into consideration, and therefore may not be sufficient to remove all pathogenic microbes from the water. In Danish farms, the application of these disinfection chemicals is widely used (Pedersen and Henriksen, 2011) but the assessment of the efficiency of these treatments are often based on normal observation on the water quality and on the fish health improvements. The implications of chemical disinfection on RAS microbial water quality needs to be elucidated in order to optimize water treatment procedures and for this Bactiquant® or other fast response method could be useful.

In general, there is scarce information regarding the potential regrowth effects that these therapeutants - or general disinfection practices - may have on microbial water quality when applied under RAS operational conditions. How the microbial water quality dynamics within RAS may be affected by the application of continually, pulse or daily treatments, still remains to be investigated.

Monitoring tools for direct assessment of disinfection procedures within RAS, are now available and it is presumed that their application will contribute to provide new information, and a better understanding, for the creation of practical guidelines regarding disinfection practices.

3.7 Practical aspects of maintaining/controlling bacterial abundance in RAS

Recirculating aquaculture systems are complex and differ from each other in terms of bacterial abundance and composition (Blancheton et al., 2013). Operating a RAS requires knowledge and experience, good observation capacity, and good routines. To maintain biostable water quality in terms of nutrient levels and bacterial abundance different strategies can be considered.

Start-up in RAS

The start-up of a RAS is important due to the fragile colonization of the slow-growing nitrifying bacteria. Feeding procedures must be carefully planned with measurements of TAN, nitrite and nitrate to identify when the nitrification process in the biofilter has started. Addition of NH_4Cl can be added before insertion of fish to reduce the bacterial colonization time hence speeding up the biofilter.

Water quality management

Water quality monitoring and management is of vital importance within RAS. Given the species of interest, various recommended threshold levels exist. Severe changes in pH (and thereby alkalinity), oxygen saturation or salinity will destabilize the bacterial communities in the biofilter and in the water.

Maintenance Practices

Maintenance practices can have a large effect on the resulting water quality, partly by avoiding catastrophic events and reducing fluctuations. Fast removal of moribund or dead fish as well as uneaten feed will reduce the risk of sudden blooms of potentially pathogenic bacteria. For example timely/regular biofilter backwash can be a central issue in fixed bed biofilters as well as drumfilter maintenance and are required to ensure optimal performance of both units present in the majority of the RAS. Unwanted biofilm growth in degassing units or on other surfaces, i.e. pipes and tanks, can also be a long-term detrimental for a RAS and lead to uncontrolled sources of organic matter and related microbial communities/biofilm.

Microbial water quality assessment

To assess changes in bacterial abundance, first step is to establish a baseline in terms of standardized measurements over a prolonged period (Besmer and Hammes, 2016). These measurements could be turbidity measurements or even visual changes by using a Secchi disk to assess visibility. Other reliable measures, ideally fast and accurate methods are needed for evaluating development and optimization of disinfection. Methodologies as Bactiquant® and HP degradation assays could provide valuable support regarding these practices.

4. Conclusions and Future perspectives

Though there is awareness of the different factors that influence microbial water quality, there is still a lack of knowledge on how the interactions of these factors influence the dynamics of bacteria in RAS. This PhD study aimed to address some of these interactions by the application of a set of new measuring tools. The main conclusions were:

Previous studies have shown the possibility to operate experimental systems up to steady state in terms of nitrate, organic matter and particles in the water under constant conditions (e.g. feed and make-up water). Large parts of this study was conducted under experimental constant conditions in RAS, where bacterial assessment showed that microbial water quality stabilization can be evaluated according to the activity from both free-living and particle-associated bacteria or it can consider the abundance of free-living bacteria only. Stabilization based on bacterial activity – assessed using Bactiquant® and HP degradation assay - showed to be highly associated with organic matter accumulation within the systems (COD_{PART}), whereas stabilization of the free-living bacteria – assessed by FCM - reflected a high association to the stabilization of the dissolved fraction of the organic matter (COD_{DISS}/DOC) (**Paper I and II**; Fig. 27). Although the major part of bacteria are probably particle-associated also in intensive RAS, they are not necessarily the only /the most important ones for RAS operation. In order to assess whether the particle-associated or the planktonic bacteria are most important for fish health and/or RAS rearing conditions more studies are needed.

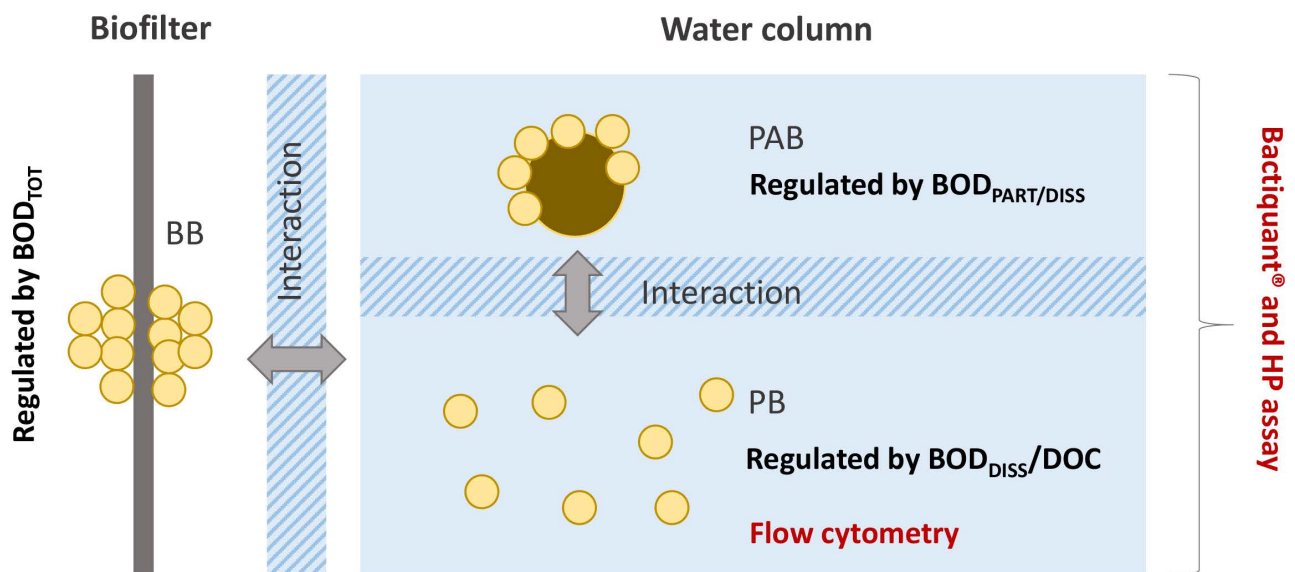


Fig. 27: Scheme indicating two main interactions: a) interaction between bacteria in biofilter (BB) and bacteria in the water column, and b) interaction between particle-associated bacteria (PAB) and planktonic bacteria (PB). The methods that assess the different parts of bacteria in the water column are indicated in red.

Feed loading is an important parameter having significant impact on RAS water quality. Further documentation was provided in **Paper II**, demonstrating how this parameter affects microbial water quality. The major conclusion from this study was that by increasing feed loading, chemical parameters as nitrate generate an immediate response whereas bacteria in the water column responded with some delay. This delay was presumed to be due to the buffer capacity exerted by the biofilter. This was confirmed in **Paper III**. Bacterial abundance (free-living bacteria) responded faster to increased feeding than bacterial activity. It was speculated whether the delay in bacterial activity could be related to particle entrapment – a known feature of fixed bed biofilters. When feeding was stopped bacterial abundance had a decline clearly related to dilution. However, after seven weeks a concentration of 10^6 cell/ml was still found in the RAS water. In regard to bacterial activity only limited changes were observed when feeding was stopped. The results suggests that transient feed stop do not necessarily have a high impact on microbial load in intensive and matured RAS.

Several studies have indicated that additional carbon source input can affect microbial water quality, particularly in RAS. In an attempt to elucidate some of these speculations, the study described in **Paper III** was performed. The main conclusions of this study were that bacteria in the water phase grow according to the expansion of the carrying capacity provided by adding a dissolved, easily degradable substrate, even in biological stable/matured systems. The results confirmed the suggestion of a biofilter buffering capacity consuming the additional carbon source added leaving bacteria in the water phase unchanged/unaffected. This biofilter effect has to be considered in future monitoring strategies for microbial water quality in the water phase in order not to misinterpret results. On the other hand, considerations towards assessing biofilter growth dynamics could lead to a better understanding on this buffering effect. This study suggests that mature biofilters may function as great backups in case of feed spill or solid removal failures. Biofilters in RAS are generally designed according to the maximum feed capacity and could be able to serve as buffers in such situations.

Based on methodologies applied within this study, it can be concluded that FCM is well suited for enumeration of free-living bacterial cells. However, this method cannot be applied directly for enumeration of bacteria attached to particles unless pre-treatment is provided. Flow cytometry applied to RAS water in this study provided new information on bacterial fingerprints, demonstrating development and dynamics of bacterial population over time that have reported for different aquatic environments but not for RAS. Further investigation on water fingerprints with FCM accompanied by sequencing tools could contribute to a better understanding of bacterial dynamics and diversity in RAS water.

Bactiquant® and HP degradation assay are new, promising tools to assess microbial water quality within RAS. Both provided a broader and more complete picture as they encompass particle-associated as well as free-living bacteria. Coulter counter measurements were found to provide important information for better understanding of the different microbial parameters. Also, the organic matter parameters measured (COD and BOD₅) provided valuable information.

Many studies have provided information to understand the importance of factors affecting general microbial communities within RAS. However, there is still a long way to go before we fully understand the dynamics of bacteria in the water phase of RAS. Biofilters bacterial communities have the capacity to suppress the response of bacterial in the water (**Paper II and III**) and this interaction between biofilter and water phase bacterial communities requires further attention to develop better monitoring procedures and to select the

correct tools. Trial setups with different types of biofilters are required to study their effects on microbial water quality dynamics and the assessment in the water column.

A necessity for the industry is a constant development of improved disinfection practices. Efficiency in disinfection procedures can only be achieved by the use of a reliable assessment tool, for example the ones proposed in this study. Moreover, future survey on full-scale RAS will increase the knowledge on operational baselines of different systems and could identify factors causing changes in bacterial dynamics and stability within a system.

Future studies could also consider sampling at short intervals to provide detailed, temporal resolutions on diurnal variations in microbial water quality in RAS.

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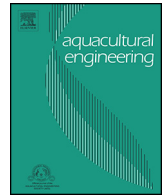
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Papers

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Bacterial activity dynamics in the water phase during start-up of recirculating aquaculture systems

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ABSTRACT

Microbial water quality in recirculating aquaculture systems (RAS) is important for successful RAS operation but difficult to assess and control. There is a need to identify factors affecting changes in the bacterial dynamics – in terms of abundance and activity – to get the information needed to manage microbial stability in RAS. This study aimed to quantify bacterial activity in the water phase in six identical, pilot scale freshwater RAS stocked with rainbow trout (*Oncorhynchus mykiss*) during a three months period from start-up. Bacterial activity and dynamics were investigated by the use of a patented method, BactiQuant®. The method relies on the hydrolysis of a fluorescent enzyme-substrate and is a rapid technique for quantifying bacterial enzyme activity in a water sample. The results showed a forty-fold increase in bacterial activity within the first 24 days from start-up. Average BactiQuant® values (BQV) were below 1000 at Day 0 and stabilized around 40,000 BQV after four weeks from start. The study revealed considerable variation in initial BQV levels between identically operated and designed RAS; over time these differences diminished. Total ammonia nitrogen, nitrite and nitrate levels were very similar in all six RAS and were neither related to nor affected by BQV. Chemical oxygen demand (COD) and biological oxygen demand (BOD₅) were highly reproducible parameters between RAS with a stable equilibrium dynamic over time. This study showed that bacterial activity was not a straightforward predictable parameter in the water phase as e.g. nitrate-N would be in identical RAS, and showed unexpected sudden changes/fluctuations within specific RAS. However, a bacterial activity stabilization phase was observed as systems matured and reached equilibrium, suggesting a successive transition from fragile to robust microbial community compositions.

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1. Introduction

Good water quality is vital to ensure aquaculture conditions that favor healthy fish and promote optimal growth (Eding et al., 2006; Timmons and Ebeling, 2007). Water quality assessment is often limited to a set of traditional physical and chemical parameters such as temperature, pH, oxygen and salinity. Recirculating aquaculture systems (RAS) are operated at high feed loading (restricted make-up water) and high fish densities requiring regular monitoring of parameters like ammonia/ammonium, nitrite, nitrate, pH, oxygen and carbon dioxide content (Dalsgaard et al., 2013). These measures are included in most commercial RAS management plans but the available set of monitoring tools is not complete. Sudden anomalies as altered fish behavior or increased mortality can occur

under apparently safe conditions, leaving a question whether some microbial related quality parameters are currently overlooked.

In RAS water, small particles accumulate (Patterson et al., 1999; Fernandes et al., 2014) due to insufficient mechanical removal and partial disintegration promoting microbial growth (Gerardi, 2006). High feed loading and long hydraulic retention time also favors microbial growth (Blancheton et al., 2013; Rurangwa and Verdegem, 2015). The implications of concomitant changes in bacterial water quality have not been thoroughly described. In 1993, a study of Vadstein et al. defined the term “microbial maturation” of the water based on “the r and K selection theory” of Andrew and Harris (1986), which distinguishes between opportunists (r-strategists; fast growing specialists) and non-opportunists (K-strategists; slow growing specialists) in the development of an ecosystem. The mature water concept assumes that water dominated by K-strategists should inhibit blooms of r-strategists on the skin and gut surface of fish larvae (Skjermo et al., 1997). Later studies have shown that RAS can have a more stable microbial com-

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munity composition favoring slow growing, competitive bacteria (K-strategy selection; Attramadal et al., 2012a).

Fluctuations in the availability of easily biodegradable substrates favor the proliferation of fast growing r-strategists bacterial species, and it is likely that increased bacterial abundance may have direct (proliferation of opportunistic pathogens) or indirect (O_2/CO_2 fluctuations, gill lamellar irritation, initiate algae bloom) detrimental effects on the reared species (Wold et al., 2014). For these reasons, microbial water quality – in terms of bacterial abundance and activity – is considered an important factor for RAS management and performance (Michaud et al., 2009). It is difficult to systematically monitor and quantify microbial water quality and evaluate the result due to the lack of a fast, robust, and reliable assessment method that can cope with RAS management requirements. Nowadays, evaluation of microbial water quality deterioration relies on simple management observations such as ceased feeding behavior or sudden mortalities, which often provide a delayed response to prolonged conditions of suboptimal water quality (Fu et al., 2015).

Two major microbial niches exist in RAS: surface attached fixed growth (biofilter carrier media and tank surfaces); and suspended microorganisms in the water column (Crab et al., 2007; Blancheton et al., 2013). The bacteria can be divided into two groups: autotrophs and heterotrophs. Ideally, autotrophic bacteria are fixed in the biofilter compartments oxidizing ammonia and nitrite, whereas heterotrophic bacteria are fixed in biofilms as well but also to suspended particles in the water, degrading dissolved and particulate organic matter (Blancheton et al., 2013; Rurangwa and Verdegem, 2015). Nitrifying biofilm processes are well described (Henze et al., 1997; Ebeling et al., 2006), whereas microbial dynamics in the water phase of RAS is still to be investigated.

Recent studies have investigated suspended, free living bacteria at a class-level (Michaud et al., 2009), phyla-level (Cytryn et al., 2003; Schreier et al., 2010), or according to their electron acceptor/donor role (Gerardi, 2006) as heterotrophs (Leonard et al., 2000) and autotrophs (Blancheton and Canaguier, 1995). Other aquaculture related studies have looked at carbon and energy sources available for microbes in RAS and the effects on the microbial water quality. Zhu and Chen (2001) and Michaud et al. (2006) showed that at higher C/N ratios, higher concentrations of free bacteria were found in the water phase. Similarly, Fu et al. (2015) demonstrated enhanced growth rates of different bacterial species as the chemical oxygen demand (COD) in water increased, turning ratios of carbon and nutrients into an indirect descriptor of microbial water quality.

From a quantitative point of view, a large number of studies in aquaculture have relied on traditional cultivation-based techniques such as colony forming units (CFU) for estimating microbial abundance (Skjermo et al., 1997; Salvesen et al., 1999; Leonard et al., 2002; Sugita et al., 2005; Michaud et al., 2009; Attramadal et al., 2012b). A main drawback of this method is that it is laborious and time consuming to perform, and provides a delayed response (taking 2–3 days). Furthermore, it only quantifies certain groups of bacteria able to grow on the substrate, and a substantial discrepancy between cultivable cell count and total cell count is therefore often observed (Staley and Konopka, 1985; Berney et al., 2008; Schreier et al., 2010). Other techniques like ATPase activity (Vang et al., 2014), incorporation of leucine as a measure of heterotrophic bacteria biomass production (Kirchman, 1993; Attramadal et al., 2014), and direct microscopic enumeration with stained cells have also been used to quantify bacteria (Porter and Feig, 1980; Sugita et al., 2005; Michaud et al., 2006; Wietz et al., 2009). A methodology such as flow cytometry has been applied as well for counting bacteria cells in water with high precision (Attramadal et al., 2014). A main hindrance for all these methods is, however, that they are

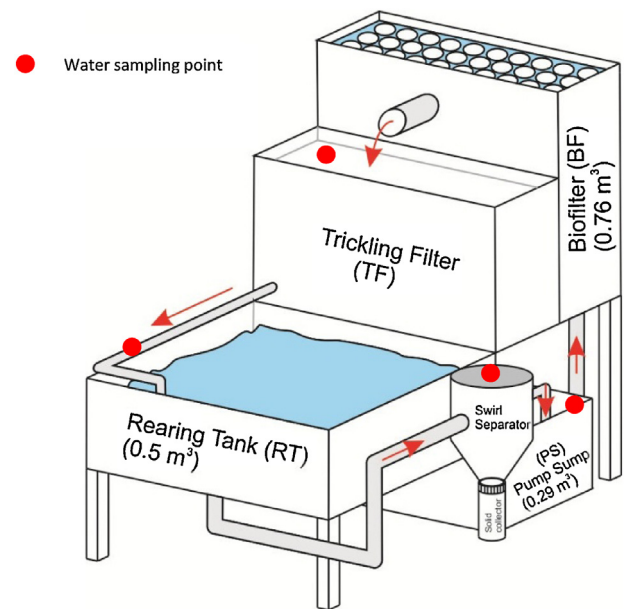


Fig. 1. A drawing of one of the recirculating aquaculture systems (RAS) used including a description of the different components in the system. Arrows indicate the water circulation direction and dots are sampling points.

complicated to perform and interpret under commercial aquaculture conditions.

In every RAS, the first microbial fingerprint occurs during the biological start-up where the system and biofilter media becomes colonized by bacteria. This phase lasts from 4 to 6 weeks, and the colonization is majorly dominated by heterotrophic and nitrifying bacteria (Hagopian and Riley, 1998). Colonization of nitrifiers in new RAS leads to a well-described, transient total ammonia nitrogen (TAN) and nitrite bell-shaped concentrations in the water phase followed by nitrate accumulation (Timmons and Ebeling, 2007). In this phase, colonization by heterotrophic bacteria causes degradation and accumulation of various particulate and dissolved organic matter/waste products. While the pattern and concentrations of the dissolved nitrogen compounds are well known and more or less predictable, bacterial dynamics in the RAS water phase during start-up is unknown. The rate of development of bacteria, their fluctuations and time span to reach steady state – if achieved – has not been thoroughly studied during this central phase.

The purpose of this study was to monitor and quantify microbial water quality (bacterial abundance and activity) in the water phase of six identical, separate RAS during start-up. By applying a new patented, culture independent method, the microbial water quality was measured on a weekly basis for a three month period to test the hypothesis of microbial succession from fluctuating to more stable conditions.

A number of classical chemical water quality parameters were concomitantly measured to evaluate system performance and investigate correlations with bacterial activity and dynamics.

2. Material and methods

2.1. System and experimental setup

The investigation was carried out in six separate but identical pilot scale RAS (labeled RAS 1, 2, etc.) each with a total volume of 1.7 m³ (Fig. 1). Each RAS was composed of a rearing tank of 500 l, a swirl separator, a pump sump, a submerged fixed-bed biofilter, and a trickling filter as described in Pedersen et al. (2012).

The systems had previously been used for other experiments but was emptied and thoroughly cleaned in all compartments to obtain similar starting conditions. Municipal tap water was added to all RAS, and dissolved ammonium-chloride was added at an approximately concentration of 2 mg TAN/l during a four weeks period to accelerate the initiation of the nitrification processes.

Rainbow trout (*Oncorhynchus mykiss*) were brought from a local farmer (Mark Moelle dambrug, Nykoebing Mors, Denmark) and kept in quarantine at 15 ppt salinity for four weeks to eliminate potential parasite transmission to the systems. At day 0, each RAS were stocked with 20 kg fish (individual weight of 54 ± 1.16 g/fish) corresponding to a density of 40 kg/m³ per tank. Fish were fed with commercial feed (EFICO Enviro 3 mm; Biomar, Denmark). During the first two weeks of the trial fish in each tank were given a fixed ration of 125 g/day, and from day 15 onwards a fixed ration of 250 g feed per tank/day was given throughout the remaining part of the three month trial. A fixed amount of make-up water (80 l/system/day) was added every morning during a period of 10 min. The make-up water exchange corresponded to 4.7% of the system volume per day and resulted in a hydraulic retention time of 3 weeks in the system. Feed loading, expressed as kg feed per m³ make-up water (Pedersen et al., 2012) was 1.56 kg feed/m³ (day 0–14) and 3.13 kg feed/m³ from day 15–93. The water flow to the rearing tank was 1.5 m³/h, and the flow from the pump sump to the biofilters was 3 m³/h. Excess water from the trickling filter overflowed back to pump sump reservoir. The biofilters were not backwashed during the trial period.

2.2. Daily management

Fish were fed from 09:00 to 15:00 every day by the use of belt feeders. Large solids/particulate matter/fecals were collected in a solid collector placed at the bottom of the swirl separator (Fig. 1) and were removed every day and checked for any uneaten pellets. Temperature, dissolved oxygen and pH were measured with Hach HQ40d instruments (Hach Lange GmbH, Düsseldorf, Germany) at the outlet of the tanks. Fish were inspected daily and any mortality was recorded. The pH was kept within the range 7.2–7.5 by daily addition of sodium bicarbonate equivalent to 20% of the added feed (i.e., 25 and 50 g/day from day 0 to 14 and day 15 to 93, respectively) to compensate for the alkalinity loss due to nitrification. The water temperature ranged from 16 to 18 °C during the three months of trial, and diurnal variations were less than 0.5 °C. Oxygen concentrations were maintained at 7–7.5 mg/L by aeration and addition of pure oxygen with diffusers in the rearing tanks. The daily photoperiod was from 7:30 to 22:00.

2.3. Water sampling and analysis

Water samples were analyzed for concentrations of TAN, nitrite, nitrate, total and dissolved biological oxygen demand (BOD_{5-TOT}; BOD_{5-DISS}), total and dissolved chemical oxygen demand (COD_{TOT}; COD_{DISS}), bacterial activity, and particle size distribution with a lower level threshold for small particles set at 5 µm (Brinker and Rösch, 2005; Fernandes et al., 2015). Particulate COD and BOD₅ (COD_{PART}; BOD_{5-PART}) were calculated by subtracting the dissolved fraction (COD_{DISS}; BOD_{5-DISS}) from the total fraction (COD_{TOT}; BOD_{5-TOT}). In order to obtain more information in relation on the organic matter characterization in RAS water, the biodegradability index was calculated. This index takes into consideration the ratio between BOD_{5-TOT} and COD_{TOT}. According to Srinivas (2008), if BOD_{5-TOT}/COD_{TOT} is >0.6 then the organic matter is easy biodegradable, if BOD_{5-TOT}/COD_{TOT} is between 0.3 and 0.6 the organic matter is average biodegradable, and if BOD_{5-TOT}/COD_{TOT} is <0.3 the organic matter is not easily biodegradable.

Samples were taken as grab samples from the tank outlet at the same day for all systems. Sample treatment and processing are presented in Table 1. BOD₅, bacterial activity, and particle distributions were measured immediately after taking water samples. Subsamples for the remaining analyses were filtered or conserved and finally stored at 4 °C for later analysis. Bacterial activity in the water phase was assessed using a patented method called BactiQuant® (Mycometer, Denmark), which is an indirect measure of microbial enzyme activity. The microbial enzymes in a water sample hydrolyze a synthetic fluorescent enzyme-substrate, releasing fluorophores that are subsequently quantified with a fluorometer. Following the manufacturers instruction, a water sample of 10 ml was filtered immediately after sampling and allocated a fixed period of 30 min to react with the fluorescent reagent before measurement as described by Reeslev et al. (2011) and Pedersen et al. (2017).

Water samples were collected from the rearing tank outlet (RT) of the 6 RAS twice a week in the first four weeks of the experiment for some parameters and thereafter once a week as specified in Table 1. In three of the six RAS, additional samples were taken from the outlet of three additional compartments in the systems (Fig. 1): pump sumps (PS), after fixed bed biofilters (BF), and after the trickling filters (TF) for approximately one month (6 sampling events from day 9 to day 51 of the experiment). This was done in order to examine any variation in the dissolved nitrogen compounds, particles, and bacterial activity between the different compartments within each system. Samples for other water quality parameters such as BOD₅ and COD were taken from the RT only.

2.4. Fish performance assessment

According to *a priori* experimental planning, fish performance assessment was done at the beginning (day 0) and at day 59 of the trial to check fish growth under the given conditions. Growth performance was assessed by calculating the feed conversion ratio (FCR) and the specific growth rate (SGR) according to Eqs. (1) and (2) (Hopkins, 1992), respectively.

$$FCR = \frac{\text{feed given (kg)}}{\text{biomass gained (kg)}} \quad (1)$$

$$SGR \left(\frac{\%}{\text{day}} \right) = \frac{\ln \left(\frac{W(t_i)}{W(t_0)} \right)}{(t_i - t_0)} \times 100 \quad (2)$$

where $W(t_i)$ refers to the total biomass at day 59, $W(t_0)$ is the total biomass at the start (day 0) of the trial, and $(t_i - t_0)$ is the total days of the relevant growth period.

2.5. Statistical analysis

Mean ± standard deviation (SD) is presented for the different parameters measured. Coefficient of variation (CV (%); CV = standard deviation/mean × 100) was calculated to compare biomass variation between systems at the start and end of the experiment. One-way analysis of variance (ANOVA) was applied to test for differences between RAS over time, treating sampling days as replicates for each system. One-way ANOVA on ranks (Kruskal-Wallis) was performed for data that violated the homoscedasticity assumption. A probability level of 0.05 was used. Statistics were done with the software program JMP7 (JMP® Version 7.0.1. SAS Institute Inc., 2007).

Table 1

Water quality parameters and analytical methods applied.

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical Method	Reference	Frequency of measurement
Temperature, pH, dissolved Oxygen	Temp., pH, O ₂	°C, pH units, mg/L	N/A	Hach HQ40d instrument, Hach Lange, Germany	N/A	Daily
Chemical oxygen demand	COD _{TOT}	mg O ₂ /L	Unfiltered + acid addition and kept at 4 °C.	LCK 414 and LCK 314, Hach Lange, Germany	ISO 6060:1989	Once a Week ^a
Dissolved chemical oxygen demand	COD _{DISS}	mg O ₂ /L	Filtered at 0.22 µm and kept at 4 °C.	LCK 414, Hach Lange, Germany	ISO 6060:1989	Once a Week ^a
Particulate chemical oxygen demand	COD _{PART}	mg O ₂ /L	N/A	COD _{PART} = COD _{TOT} – COD _{DISS}	N/A	Once a Week ^a
Biochemical oxygen demand of 5 days	BOD _{5-TOT}	mg O ₂ /L	Unfiltered	Potentiometry/O ₂ probe (WTW Oxi 340i)	ISO 5815:1989	Once a Week
Dissolved biochemical oxygen demand of 5 days	BOD _{5-DISS}	mg O ₂ /L	Filtered at 1.6 µm	Potentiometry/O ₂ probe (WTW Oxi 340i)	ISO 5815:1989	Once a Week
Particulate biochemical oxygen demand of 5 days	BOD _{5-PART}	mg O ₂ /L	N/A	BOD _{5-PART} = BOD _{5-TOT} – BOD _{5-DISS}	N/A	Once a Week
Total ammonia nitrogen	TAN	mg/L	Filtered at 0.22 µm. Conserved at 4 °C.	Colorimetry	DS 224	Once a Week ^a
Nitrite	NO ₂ -N	mg/L	Filtered at 0.22 µm. Conserved at 4 °C.	Colorimetry	DS 223	Once a Week ^a
Nitrate	NO ₃ -N	mg/L	Filtered at 0.22 µm. Conserved at 4 °C.	Colorimetry	ISO 7890-1:1986	Once a Week ^a
Particle size distribution	PSD		Unfiltered. Processed immediately	Optical Particle Counter (AccuSizer™ 780 SIS, Particle Sizing Systems, United States of America)	Fernandes et al. (2014)	Once a Week
Bacterial Activity	BactiQuant Value	BQV	Unfiltered. Processed immediately	BactiQuant®	Mycometer A/S, Denmark	Once a Week ^a

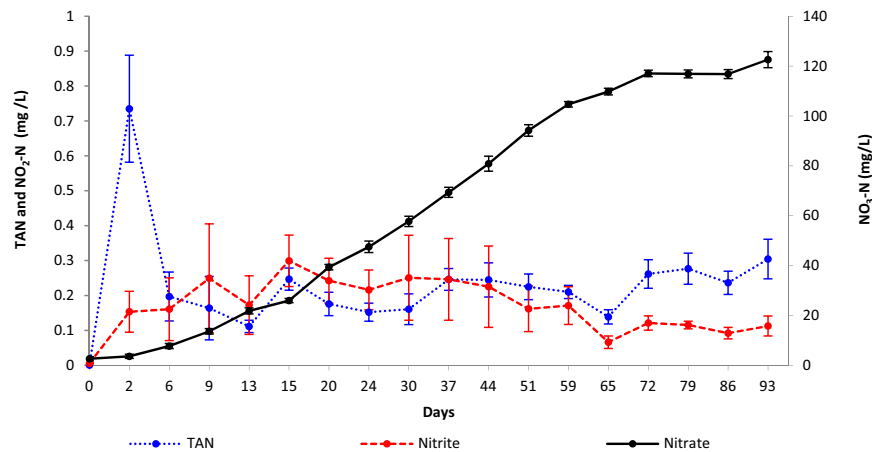
^a From day 0 to day 24, these parameters were sampled twice a week.

Fig. 2. Mean concentrations of dissolved nitrogen compounds in the 6 systems (mean \pm SD, $n = 6$). Daily feeding was 125 g/day in each recirculating aquaculture system (RAS) from day 1–14, hereafter increased to 250 g/day in each RAS. At day 59, feeding was stopped for two days to assess the biomasses. No significant differences were found between RAS/systems for total ammonia nitrogen (TAN) and NO₃-N ($P = 0.660$ and 0.999 , respectively) averaged over the whole sampling period ($n = 18$), whereas significant variation in NO₂-N ($P < 0.01$) between RAS was observed. Water samples were collected at the rearing tank outlet of each RAS.

3. Results

3.1. Dissolved nitrogenous compounds

Fig. 2 shows the mean concentration of TAN, nitrite, and nitrate in the 6 systems over the three months of the experiment. All biofilter performances were consistent over time showing rapid

stabilization of TAN and nitrite levels after start-up. Mean nitrate-N build-up reached 123 ± 3.22 NO₃-N mg/L after three months. No significant differences in TAN ($P = 0.660$) or nitrate ($P = 0.999$) concentrations were found between RAS whereas NO₂-N differed significantly between RAS ($P < 0.01$), as also implied by the large variation in this parameter in the initial phase.

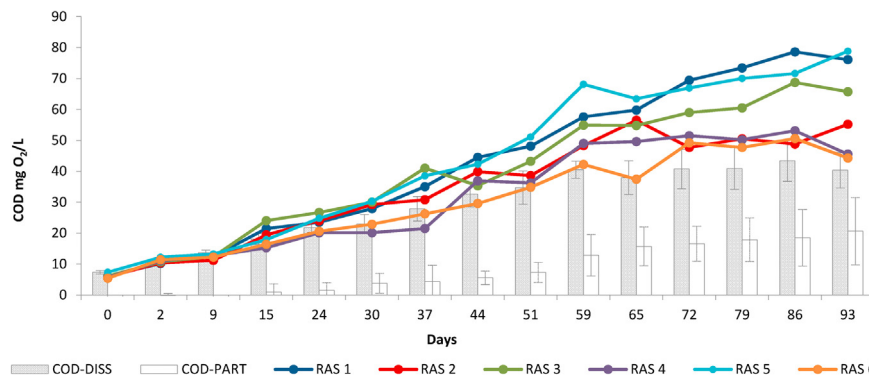


Fig. 3. Concentrations of total chemical oxygen demand (COD_{TOT}) in all six recirculating aquaculture systems (RAS) over a period of three months (lines). Mean dissolved COD (COD_{DISS}) and particulate COD (COD_{PART}) concentrations are shown in grey and white bars with their respective standard deviations (mean \pm SD, $n=6$). Significant variations were seen between RAS for COD_{TOT} ($P=0.033$) and COD_{PART} ($P<0.01$), but not for COD_{DISS} ($P=0.079$) when averaged over the whole sampling period ($n=15$). Water samples were collected at the rearing tank outlet of each RAS.

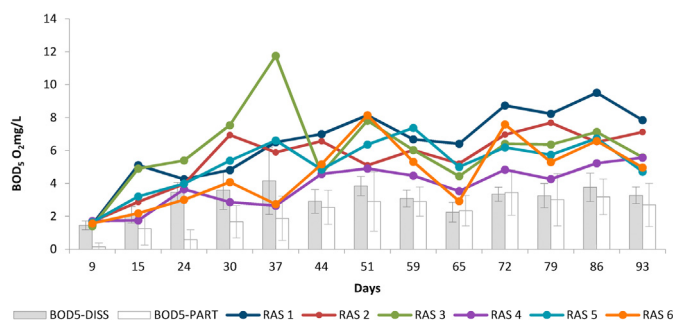


Fig. 4. Concentrations of the total biological oxygen demand (BOD_{5-TOT}) in the six recirculating aquaculture systems (RAS) (dots and lines), and mean dissolved (BOD_{5-DISS}) and particulate (BOD_{5-PART}) BOD_5 concentrations during three months trial (bars; mean \pm SD, $n=6$). All BOD_5 fractions (BOD_{5-TOT} , BOD_{5-PART} and BOD_{5-DISS}) varied significant between RAS ($P<0.01$) when compared over the whole sampling period ($n=13$). Water samples were collected at the rearing tank outlet of each RAS.

3.2. Organic matter

The organic matter content measured as COD_{TOT} increased with a constant rate for two months hereafter reaching a plateau with some variation between RAS (*i.e.*, varying from 44 to 79 mg O_2/L ; Fig. 3). Mean COD_{DISS} build up over time and was the predominant fraction of COD_{TOT} , with mean COD_{PART} being significantly lower and building up more slowly over time. When tested across the whole sampling period there were significant differences between RAS in COD_{TOT} ($P=0.034$) and COD_{PART} ($P<0.01$), whereas there were no differences in COD_{DISS} ($P=0.079$) between systems. At the end of the trial, COD_{DISS} constituted 66% and COD_{PART} 34% of COD_{TOT} .

BOD_{5-TOT} concentrations measured at day 9 after experimental start-up were very similar in all of the six RAS (1.58 ± 0.12 mg O_2/L ; Fig. 4), with the majority present as BOD_{5-DISS} . However, all BOD_5 fractions were found to be significantly different between the systems ($P<0.01$) when averaged over the whole trial (13 sampling days). On day 15, BOD_{5-TOT} concentrations already started to diverge between systems. At the end of the experiment, RAS 1 reached a BOD_{5-TOT} concentration of 7.84 mg O_2/L while the lowest concentration (RAS 5) was 4.96 mg O_2/L . Mean BOD_{5-DISS} increased from 1.44 ± 0.27 to 3.08 ± 0.52 mg O_2/L at day 9 and 93, respectively, and mean particulate BOD_{5-PART} increased from 0.14 ± 0.24 to 2.9 ± 0.89 mg O_2/L in the same period. At the end of the experiment the mean dissolved and particulate fractions constituted 55 and 45%, respectively, of the mean total BOD_5 . The mean biodegradability index for BOD_{5-TOT}/COD_{TOT} was 0.1 ± 0.03 .

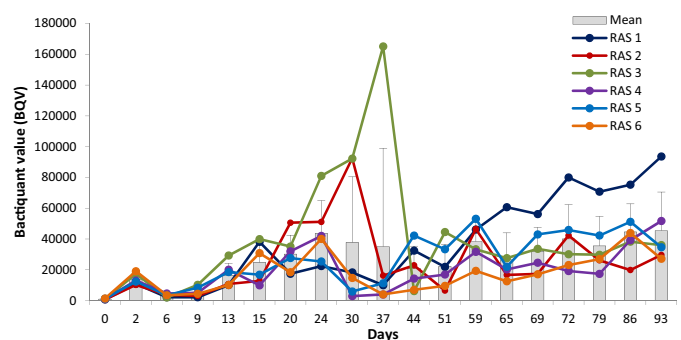


Fig. 5. Bacterial activity measured as BactiQuant® values (BQV) in the six recirculating aquaculture systems (RAS) during the three months trial. Mean BQV is shown as bars with their respective standard deviation ($n=6$). BQV varied significant between RAS ($P<0.01$) when compared over the whole sampling period ($n=19$). Water samples were collected at the rearing tank outlet of each RAS.

3.3. Bacterial activity and abundance

Total bacterial activity, quantified as BQV, displayed irregular patterns over time and between systems (Fig. 5). Four out of the six RAS were found to have similar patterns/development of BQV, whereas two RAS (system 2 and 3) deviated by transiently elevated BQVs. A one-way ANOVA revealed that bacterial activity was significantly different between RAS ($P<0.01$) when comparing BQV averaged over the whole culture period.

Bacterial activity measured at different positions within three of the six RAS is shown in Fig. 6. No differences were found for BQV within each RAS ($P=0.955$) when averaged over time (*i.e.* no effect of sampling position). However, BQV levels differed significantly between RAS when averaged over the sampling period, as mentioned above.

3.4. Particles

Particle numbers ranged from 691 ± 457 particles/ml (mean \pm SD, $n=6$) at day 9 to 6794 ± 2282 particles/ml at day 59. At day 9, RAS 2 had a higher number of particles compared to the rest of the RAS which were quite similar between them (1598 versus 509 ± 118 particles/ml in RAS 2 and the other five RAS, respectively). At day 59, the numbers of particles were similar in all RAS except RAS 6 (2779 versus 6951 ± 2515 particles/ml in RAS 6 and the other five RAS, respectively). Particle numbers did not vary between different compartments within the same RAS (one-way ANOVA; $P>0.05$ for all three RAS). In numbers,

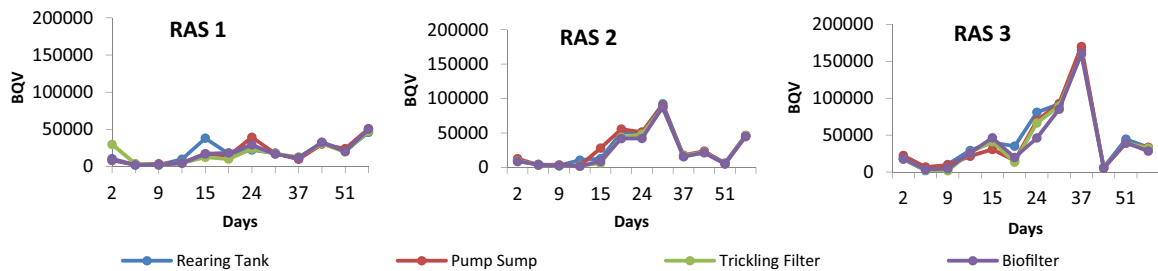


Fig. 6. Bacterial activity measured as BacteriQuant® values (BQV) in four different compartments/positions within each of three separate recirculating aquaculture systems (RAS). No statistical difference was seen between compartments of the same RAS ($P=0.955$; $n=6$) whereas BQV were significantly different ($P<0.01$) in RAS 3 compared to the other two RAS when averaged over the sampling period ($n=6$) independently of the sampling position.

fine particles ranging from 5 to 20 μm dominated the systems constituting 96% of total counts.

3.5. Fish growth

Fish biomass increased from 20.32 ± 0.14 kg to 32.6 ± 0.43 kg (mean \pm SD, $n=6$) during the first 59 days of the trial. No difference in fish biomass was found between the six RAS, neither at start-up (day 0) ($\text{CV}=0.70\%$) nor at biomass assessment (day 59) ($\text{CV}=1.32\%$). Mean FCR was 1.10 ± 0.03 and mean SGR was $0.82 \pm 0.01\%$ /day. Overall, mortality was less than 1% (in numbers) throughout the three months experiment and was similar in all six RAS.

4. Discussion

4.1. Chemical water quality parameters

When starting up the RAS in the present study, introduction of fish and addition of feed immediately caused changes in the water quality. All six RAS showed consistently low TAN and nitrite concentrations after two weeks from start of feeding. The fast colonization and limited accumulation of TAN and nitrite was in this particular case probably achieved because the RAS, although thoroughly cleaned, had been previously in operation and because NH_4Cl was supplemented for biofilter activation prior to addition of fish.

Colt et al. (2006) exemplified some definitions of steady state in RAS studies involving nitrification stable TAN and nitrite concentrations, and called for other definitions to further standardize such studies. Here, we considered steady state and system equilibrium regarding dissolved N-fractions to be achieved when nitrate accumulation ceased and constant nitrate levels were obtained as a consequence of the fixed feed load and the fixed supply of make-up water. After two months, such expected stabilization of the nitrate concentrations (around 120 mg $\text{NO}_3\text{-N/l}$) was achieved in line with previous studies (Pedersen et al., 2012). At this point, a nitrogenous equilibrium was established and N-excretion (input) matched the N-removal achieved by the make-up water addition (output), consequently leading to a stable nitrate concentration. At the same time, ammonium and nitrite oxidizing bacteria performed stable, generally leading to fixed levels and ratios between the two groups of nitrifiers with a higher substrate dependent removal rate of the nitrite oxidizing bacteria (cf. data from day 65 and onwards; Fig. 2).

Organic matter, measured as COD, increased in a similar way as nitrate. Initially, a linear increase was observed which then ceased and plateaued after two months, an observation in line with previous studies (Fernandes et al., 2015). Furthermore, a pronounced accumulation of dissolved vs. particulate COD was found. The dynamics of the readily bio-degradable organic matter (BOD_5) and the concentrations at steady state were also similar to previous

findings (Fernandes et al., 2015; Hambly et al., 2015), and equilibrium was seemingly achieved after 30 days. Sudden system specific changes in BOD_5 were observed, however, not related to any specific event (see Section 4.2). The mean biodegradability index for $\text{BOD}_{5\text{-TOT}}/\text{COD}_{\text{TOT}}$ was 0.1 ± 0.03 which, according to the classification proposed by Srinivas (2008), classifies the organic waste in the RAS water as not easily biodegradable. This means that the soluble fraction of the organic matter in the water was composed of high amounts of hard to degrade and/or nonbiodegradable substances. Dalsgaard and Pedersen (2011) were the first study to use the biodegradation index to characterize aquaculture organic matter in the water column in form of dissolved ratio ($\text{BOD}_{5\text{-DISS}}/\text{COD}_{\text{DISS}}$) and particulate ratio ($\text{BOD}_{5\text{-PART}}/\text{COD}_{\text{PART}}$). In this present study, the corresponding dissolved and particulate ratios (0.08 ± 0.01 and 0.18 ± 0.12 , respectively) were very low, signifying that the hard to degrade and/or nonbiodegradable organic matter was dominating in both fractions.

4.2. Bacterial activity dynamics

Increased levels of dissolved nutrients and organic matter seemed to affect bacterial activity, probably by leading to new and changing conditions for bacterial growth, likely affecting fixed film growth on surfaces as well as suspended growth in the water phase (Blancheton et al., 2013; Rurangwa and Verdegem, 2015). Changing, unstable conditions are believed to favor colonization of fast growing opportunistic bacteria (r-strategists) (Andrew and Harris, 1986; De Schryver et al., 2014). The phase is characterized by a relatively high nutrient availability per bacteria, which induces potential for fast growing opportunistic heterotrophic bacteria (Hagopian and Riley, 1998; Attramadal et al., 2012b). Most likely, the overall bacterial growth rate will eventually cease, and bacterial activities stabilize towards system equilibrium. However, it was not possible to assess if such microbial maturation occurred in the present study as no microbial community analyses were carried out.

In this experiment, we tested initial and subsequent activity dynamics of suspended bacteria during the start-up phase of RAS by creating constant conditions (i.e., identical daily feed input and make-up water addition) over a prolonged period of time. The dynamics of microbial activity (measured as BQV) were found to be more irregular and less predictable within and between RAS during the initial start-up compared to the latter part of the study. During start-up, RAS are unbalanced in terms of nutrients and organic matter meaning that these components are in constant production/degradation and accumulation within the systems. Accordingly, we presume that this may have been the reason for the substantial changes in BQV observed (Fig. 5). Eventually, this initial and dynamically fragile phase was apparently succeeded by a more dynamically robust phase with the bacterial activity stabi-

lizing after 59 days. However, random and unpredictable changes still occurred in individual RAS.

On the other hand, it must be addressed that the method applied was not able to determine potential changes in bacterial community composition, however, we speculate that the findings might well fit the theory of microbial succession in RAS, stating that fast-growing opportunistic bacteria (r-strategists) are outperformed by slower growing bacteria (K-strategists) as a consequence of constant substrate supply per bacterium (De Schryver et al., 2014; De Schryver and Vadstein, 2014).

The BactiQuant® method applied in the present study measures the bacterial hydrolase activity and is therefore an indirect measure for microbial activity (Reeslev et al., 2011). BactiQuant® values reflect the number of bacteria and the enzyme activity in the given water samples under the influence of sample volume, reaction time, and incubation temperature, which were all kept constant throughout the study. Therefore, BQV measured in the present study were related to bacterial abundance and activity only and hence directly comparable. The striking dynamics and apparent BQV outliers at specific sampling days were neither a result of the method applied nor sampling position. The implementation of multi-position sampling (Fig. 6) with identical BQV readings rules out erroneous sampling and confirms reproducibility of the method applied. The findings stress that irregular and unexplained events take place within RAS that have a substantial effect on the microbial activity in the water phase. On the

The frequency of BQV measurements did not allow scrutinizing short term changes such as abrupt release of biofilm, sudden altered fish behavior, or other events that potentially could trigger a rapid change in substrate availability and subsequent bacterial activity.

To our knowledge, this is the first study quantifying the dynamics of bacterial activity in freshwater RAS during start-up. The findings provide initial information on the dynamics in bacterial activity, and the results seem to support that water quality influences changes in microbial activity. The study illustrates that microbial activity dynamics is reasonably predictable on an overall level although sudden, irregular changes occur particularly in the start-up phase, leaving a number of open questions on causality. Future research, investigating the causal relationship between microbial activity and water quality parameters (e.g. bioavailable organic matter) can hopefully provide important further knowledge of theoretical and practical relevance to the future development of RAS (Hambly et al., 2015). Studies testing microbial activity combined with bacterial counting in different systems could perhaps shed light on mechanisms and consequences of RAS carrying capacity, bacterial regrowth potential, and effects of water treatment on system stability and associated bacterial dynamics.

5. Conclusions

Low levels of TAN and nitrite and predictable levels of nitrate were found in all six RAS during start-up and system equilibrium. In contrast, BactiQuant® measurements revealed substantial fluctuations in microbial activity over time and between RAS, especially in the initial phases. Bacterial activity, however, stabilized approximately three weeks after system start-up. The study illustrates the bacterial dynamics in RAS during start-up to the maturation phase, and emphasizes the need for more knowledge of factors (e.g. feeding loading, feed composition, fish size, temperature, disinfection, etc.) affecting microbial water quality in RAS. Further focus on the development of rapid

methods: like BactiQuant® to monitor bacterial activity are expected to provide valuable new knowledge of microbial activity and biostability in RAS, allowing RAS managers to improve and optimize corresponding operational actions.

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Changes in microbial water quality in RAS following altered feed loading

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ABSTRACT

Intensive recirculating aquaculture systems (RAS) with its hyper-eutrophic water offer ideal conditions for bacterial growth, abundance and activity, potentially affecting fish and system performance. Feed composition and feed loading in particular will have significant impact on organic and inorganic nutrients available for microbial growth in RAS. How these nutrient inputs affect and regulate bacteria in RAS water is, however, unclear. To investigate this relationship and the associated water quality dynamics, the effects of altered feed loading on microbial water quality in RAS was studied.

The study included six independent, identical pilot-scale RAS, each with a total volume of 1.7 m³ (make-up water: 80 L/day) stocked with juvenile rainbow trout (*Oncorhynchus mykiss*). All systems had been operating with constant and identical feed loading of 3.13 kg feed/m³ make-up water for a period of three months before the experiment was initiated. Three controlled levels of feed loading were established in duplicates: no feed (0 kg feed/m³), unchanged feeding (3.13 kg feed/m³), and doubled feeding (6.25 kg feed/m³). The experimental period was seven weeks, where microbial and chemical water quality was monitored weekly. Bacterial activity was measured using Bactiquant[®], and microbial hydrogen peroxide degradation. Bacterial abundance was quantified by flow cytometry, and water quality parameters by standardized methods.

The study showed that water quality as well as bacterial activity and abundance were affected by the changes in feed loading. The microbial water quality parameters, however, did not respond to feed loading changes as quickly and straightforward as the physicochemical parameters such as nitrate, chemical oxygen demand and biological oxygen demand. It was presumed that the fixed bed biofilter suppressed microbial response in the water phase. Hydrogen peroxide degradation assay proved to have considerable potential for assessing overall bacterial load in RAS water although further adjustments and standardization procedures are required.

1. INTRODUCTION

The aquatic environment in recirculating aquaculture systems (RAS) is complex, consisting of multiple biotic and abiotic water quality parameters (Timmons *et al.*, 2009) including surface associated and suspended bacteria. Feed composition and digestibility (Lam *et al.*, 2008; Blancheton *et al.*, 2013), and feed loading (Pedersen *et al.*, 2012; von Ahnen *et al.*, 2015), significantly affect the aquatic environment and nutrient abundances in RAS. Dissolved compounds and fine particulate organic matter are complicated to remove, so micro particles accumulate within the system serving as substrate and surface area for heterotrophic bacteria (Wold *et al.*, 2013; Pedersen *et al.*, 2017). These bacteria-inhabited particles will degrade further until they eventually become part of the dissolved organic fraction of the water and sustain further bacterial growth. Several studies have shown that the bacterial population in RAS is highly dominated by heterotrophic bacteria (Leonard *et al.*, 2000; Michaud *et al.*, 2009; Michaud *et al.*, 2014; Rud *et al.*, 2017) both in suspension and on surfaces. These bacteria obtain energy from the degradation of organic carbon compounds (Prest *et al.*, 2016b). They occupy available niches that could potentially be used by specific pathogenic bacteria (Attramadal *et al.*, 2012; Blancheton *et al.*, 2013). However, high abundance of these bacteria may directly or indirectly affect the fish, acting as opportunistic pathogens or competing for oxygen and potentially affecting the nitrification process as they compete for space with the autotrophic bacteria (Blancheton *et al.*, 2013, Michaud *et al.*, 2014).

In RAS, water quality parameters are controlled to maintain stable physicochemical water quality for the fish. Since bacteria are omnipresent in RAS, it is important to get a better understanding of the factors that causes changes in microbial water quality and, if possible, to understand how to regulate and control these factors in order to achieve biological stable RAS water of high quality. Monitoring tools are necessary to observe and control microbial water quality, but the tools available are generally complex or associated with a considerable time lag from water samples are taken to results are obtained (Rojas-Tirado *et al.*, 2017). Moreover, no guidelines on which critical parameters to measure exist, and the range of acceptable levels and acceptable fluctuations are not known. Therefore, there is a need for new operational tools and for establishing associated guidelines to describe and control bacterial loads in RAS water.

To contribute to this, the following experiment was conducted to describe changes in microbial water quality in terms of bacterial activity and abundance associated with changes in feed loading. Six pilot scale RAS were operated under identical conditions for three months to reach steady state before changes in feed loading were made. Three groups with different feed loading were then established and concomitant changes in water quality parameters were monitored.

73 2. MATERIAL AND METHODS

74 2.1 System and experimental setup

75 The experiment was conducted in six identical, separate pilot scale RAS each operated under constant and
76 identical conditions over a period of thirteen weeks prior to this study (Rojas-Tirado *et al.*, 2017). Following
77 the thirteen week period of fixed feed loading (FL) (250 g feed/day per RAS and 80 L/day make-up water;
78 corresponding to a feed loading of 3.13 kg/m³ day), three different levels of feed loading were allocated to
79 the six RAS (duplicate study): i) 0 kg/m³ day (FL₀) ii) 3.13 kg/m³ day (FL_{3.13}) and iii) 6.25 kg/m³ day (FL_{6.25})
80 (Table 1). The effect of changed feed loading on bacterial activity and abundance was then evaluated for
81 seven weeks (week 0 to week 7), where week 0 denotes the time of changed feed loading.

82 Details on system design and management can be found in Fig. 1 and Rojas-Tirado *et al.* (2017). Briefly, each
83 RAS was stocked with 32.4 ± 0.49 kg rainbow trout (*Oncorhynchus mykiss*) and fed daily with commercial
84 feed (EFICO Enviro 3 mm; Biomar, Denmark) from 9:00 to 15:00 by the use of a belt feeder. The photoperiod
85 was from 7:30 to 22:00. Dissolved oxygen, temperature and pH were measured on a daily basis. Oxygen
86 concentration was maintained above 80 % saturation, pH between 7.3-7.4 and, temperature 19 ± 0.3 °C.

87 Daily management of each RAS included: i) solids removal by emptying the accumulated feces in the sludge
88 collectors at the bottom of the swirl separators (Fig. 1), ii) addition of 80 L make-up water (tap-water)/day
89 per RAS (corresponding to 4.7 % of the system volume), iii) cleaning and loading of the belt feeders, and iv)
90 addition of sodium bicarbonate equivalent to 20 % of the weight of the added feed to compensate alkalinity
91 loss due to the nitrification process. The biofilters were not backwashed during the experimental period.
92 Inspection and removal of any dead or moribund fish took place on a daily basis.

93 2.2 Water sampling and analysis

94 Weekly, grab samples of 2 L water were taken from the outlet of the tanks (siphoned gently from the top of
95 the swirl separators) of each RAS at 8:00 before feeding and management routines. The selected parameters
96 used to assess the physicochemical and microbial water quality are described below and listed in Table 2 and
97 3, together with their sampling procedure, treatment and processing.

98 2.2.1 Physicochemical water quality parameters

99 Total and dissolved chemical oxygen demand (COD_{TOT} and COD_{DISS}) as well as total and dissolved biological
100 oxygen demand (BOD_{5-TOT} and BOD_{5-DISS}) were used to characterize the organic matter content in the water.
101 The particulate fraction of COD and BOD (COD_{PART} and BOD_{5-PART}) were calculated by subtracting the dissolved
102 fraction from the total (COD_{TOT} - COD_{DISS} = COD_{PART}; BOD_{5-TOT} - BOD_{5-DISS} = BOD_{5-PART}). Additional analyses
103 including total ammonia nitrogen (TAN), nitrite-nitrogen (NO₂⁻-N) and nitrate-nitrogen (NO₃⁻-N) were
104 performed on filtered samples stored at 4 °C until analysis. Table 2 specifies the sampling procedure, and
105 treatment and analysis of the water samples for each of the physicochemical water quality parameters.

106 The submerged, fixed-bed biofilters were not backwashed during the experiment but at the end of the
107 experiment (week 7) organic matter accumulated at the bottom was assessed. Six hours after stopping the
108 pumps and subsequent settling of solids in the biofilters, 80 L was collected from a bottom drain of each

109 biofilter. These six collections were each homogenized and 2 L subsamples from each were used for analysis
110 of total solids (TS) and ashes.

111 2.2.2 Microbial water quality assessment

112 *Bacterial activity*

113 Bacterial activity in the water phase was assessed by two different methods, BactiQuant® (Mycometer,
114 Hillerød, Denmark) and hydrogen peroxide degradation assay. Bactiquant measures bacterial activity
115 indirectly via a common hydrolase enzyme found within a wide range of bacteria (Reeslev *et al.*, 2011). A
116 well-defined water volume was filtered through a 0.22 µm filter, on which particle-bound and free bacteria
117 are trapped; the filter cake is then exposed to a fluorescent substrate and depending on the amount of
118 bacteria present and their activity, a quantitative fluorescent signal can be detected. The BQ values (BQV)
119 were calculated according the sample volume (10 ml), exposure time (30 min) and incubation temperature
120 (measured on site) as described by Rojas-Tirado *et al.*, (2017) and Pedersen *et al.* (2017).

121 The hydrogen peroxide (HP) degradation assay applied was derived from the principle of microbial activity
122 degradation kinetics described in Arvin and Pedersen (2015). The method quantifies the sum of enzymatic
123 degradation of HP (Hossetti and Frost, 1994) in a water sample based on the presence and activity of free
124 and particle-bound bacteria. A high bacterial activity and abundance in the water phase causes a fast HP
125 degradation where more than 20 mg HP/l can be degraded in less than one hour (Pedersen, 2013). The
126 degradation kinetics can be described as a first order reaction by the exponential decay equation: $C_t = C_0 \cdot e^{-kt}$,
127 with k being the descriptive reaction rate constant (per hour), C_0 the initial concentration of HP (mg/L), C_t the
128 concentration at time “ t ” in hours (h). Water samples were taken from the outlet of the tank from each RAS
129 and transferred to 500 ml beakers, stirred at 250 RPM at room temperature. Beakers were then spiked with
130 HP to reach an initial nominal concentration of 8 mg HP/L. Hydrogen peroxide concentrations were measured
131 2, 10, 30 and 60 minutes after HP addition by the spectrophotometric method described by Tanner and Wong
132 (1998) and modified by Pedersen and Pedersen (2012). Hydrogen peroxide degradation was measured on
133 water samples from each system for three consecutive weeks towards the end of the experiment.

134 *Bacterial abundance assessment*

135 The total number of bacterial cells was quantified by flow cytometry using a BD Accuri™ C6 Flow Cytometer
136 (BD Bioscience, San Jose, CA, USA), using staining of DNA with SYBR Green I (nucleic-acid gel stain, Molecular
137 Probes Invitrogen) and excitation with the blue laser (488 nm) (Marie *et al.*, 2005; Wold *et al.*, 2014). The
138 threshold for the FL1-A channel was set at $10^{3.25}$. Signals above that threshold were considered as bacterial
139 cells, and signals below were considered to be background signals. Water samples did not receive any
140 treatment that could provide cell detachment from particles so the data obtained can be regarded as bacteria
141 cells suspended in the water phase (“free-living bacteria”). Water samples were checked for presence of
142 phytoplankton, but none were detected.

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146 **2.3 Assessment of fish performance**

147 Fish biomass in each system was measured five weeks (week -5) before changes in feed loading and at the
148 end of the experiment (week 7). Feed conversion ratio (FCR; feed intake/biomass gain) and specific growth
149 rate (SGR) were calculated according to Hopkins (1992).

150 **2.4 Data analysis**

151 The different parameters measured are presented as mean \pm standard deviation. Data were log-transformed
152 when necessary to meet normality (normal distribution). One-way analysis of variance (ANOVA) was applied
153 to test for difference between treatments at week 0 and week 7. For data not meeting the homoscedasticity
154 assumption, the one-way ANOVA on ranks (Kruskal-Wallis) was performed. Difference in treatment means
155 were tested by Tukey's least square means test, with a significance level set at $p < 0.05$. Statistics were
156 performed using the software SigmaPlot 12.5 from Systat Software, Inc., San Jose California USA.

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174 3. RESULTS

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176 3.1 Dissolved N and organic matter

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178 TAN and nitrite remained stable at low concentrations throughout the experiment in all six RAS (Table 4).
179 Nitrate-N concentrations immediately started to diverge when feed loadings were changed (from week 1;
180 Fig. 2). Nitrate concentrations decreased from 133 ± 1 mg NO₃-N/L to 59 ± 0 mg NO₃-N/L in the water from
181 the FL₀ RAS, stayed constant for the FL_{3.13} (133 ± 0.9 to 159 ± 1.3 mg NO₃-N/L), and increased steadily in the
182 water from the FL_{6.25} RAS (133 ± 5.6 to 280 ± 11 mg NO₃-N/L).

183 The biodegradable organic matter (BOD_{TOT}) was significantly reduced by 55 % at week 7 in FL₀ (Fig. 3; $p <$
184 0.05). The BOD_{TOT} in the control RAS (FL_{3.13}) increased somewhat, peaking at 9.5 ± 1.6 mg O₂/L in week 5 and
185 ending at 6.3 ± 2.84 in week 7. In comparison, transient levels up to 20 mg O₂/L were observed in the FL_{6.25}
186 RAS, 5-6 weeks after feeding was doubled and ended up at 7.19 ± 1.6 mg O₂/L (Fig. 3). The dissolved fraction
187 of BOD in the FL₀ RAS was reduced by approximately 86 % at the end of the experiment (from 2.93 ± 0.22 to
188 0.4 ± 0.13 mg O₂/L; Table 4), which is low compared to the other two treatments (3.3 ± 0.8 and 5.8 ± 1.94 mg
189 O₂/L in the FL_{3.13} and FL_{6.25} RAS, respectively; Table 4). Decrease in dissolved BOD in the FL₀ RAS correlated
190 significantly ($r = 0.75$; $p < 0.05$; $n = 14$) with NO₃-N concentration, which is decreases due to dilution.

191 Total COD decreased by 33 % in the FL₀ RAS ending at 34.0 ± 6.3 mg O₂/L, whereas the control RAS (FL_{3.13})
192 and the FL_{6.25} RAS increased with 16 and 5 %, respectively (ended at 65.3 ± 18.7 and 82.2 ± 7.8 mg O₂/L; Table
193 4). The dissolved COD fraction (COD_{DISS}) in FL₀ RAS steadily decreased from 37 ± 2 mg O₂/L to 16 ± 1 mg O₂/L
194 (Fig. 4; Table 4), ending significantly lower ($p < 0.05$) than the other two treatment groups (33 ± 2 and $45 \pm$
195 5 mg O₂/L, respectively). The decrease in COD_{DISS} in the FL₀ RAS was highly correlated to the reduction in NO₃-
196 N ($r = 0.97$; $p < 0.0001$). The particulate COD fraction (COD_{PART}) increased transiently by 4-6 fold in the FL₀
197 RAS, reaching levels above 40 and 60 mg O₂/L in week 3-5 in the two unfed RAS (Fig. 4, a). In week 7, at the
198 end of the experiment, it was 18.25 ± 5.1 mg O₂/L compared to 32.8 ± 17.2 and 38.0 ± 2.44 mg O₂/L in FL_{3.13}
199 and FL_{6.25} RAS, respectively (Table 4).

200 The BOD_{TOT}: COD_{TOT} ratio changed from 1:8 to 1:16 in FL₀, whereas in the other two treatment groups it
201 remained stable around 1:11. The biodegradability index: BOD₅/COD (Srinivas, 2008) was thus around 0.1 in
202 all RAS during the trial, except for the FL₀ RAS ending at 0.06.

203 Dry matter content in the reject water from the standardized biofilter backwash performed the end of the
204 experiment (week 7) was positive and significantly related ($p < 0.05$) to feed loading with 1.5, 16 and 32 g/L
205 for FL₀, FL_{3.13}, and FL_{6.25} RAS, respectively. The associated ash content followed the same pattern with 0.7 g/L,
206 4 g/L, and 8 g/L in FL₀, FL_{3.13} and FL_{6.25} RAS, respectively.

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212 3.2 Microbial water quality parameters

213 3.2.1 Bacterial activity assessments

214 Bacterial activity measured by Bactiquant®, showed that the six RAS had activities in the range of $3-9 \times 10^4$
215 BQV/ml before changing the feed loadings (Fig. 5). At the end of the experiment, the bacterial activity in the
216 FL₀ RAS ($5.2-5.6 \times 10^4$ BQV/ml) was 2-4 times lower ($p < 0.05$) than in the FL_{3.13} and the FL_{6.25} RAS's ($1-1.5 \times$
217 10^5 and $1.1-1.3 \times 10^5$ BQV/ml, respectively).

218 Hydrogen peroxide (HP) degradation rates in the water from all RAS were significantly affected by feed
219 loading ($p < 0.001$). The lowest HP removal rate was measured in the water from the unfed RAS, FL₀ ($1.4 \pm$
220 0.3 mg/L reduction in HP concentration after 30 min) with a mean rate constant (k) of 0.41 h^{-1} (Fig. 6, a). The
221 water from the FL_{3.13} RAS had a 4 ± 0.36 mg/L reduction in HP concentration after 30 minutes (Fig. 6, b) and
222 a mean rate constant of $1.73 \pm 0.38 \text{ h}^{-1}$, whereas almost complete removal of HP (6 ± 0.49 mg HP/L reduction
223 within 30 min) was observed in the water from the FL_{6.25} RAS (Fig. 6, c) reaching a mean rate constant of 4.92
224 $\pm 0.86 \text{ h}^{-1}$ at the end of the trial.

225 3.2.2 Bacterial abundance

226 Flow cytometry showed that the concentration of free-living bacteria in the six RAS was in the range of $0.6 -$
227 3.8×10^7 cells/ml before changing the feed loadings. The abundance decreased from $2.0-2.4 \times 10^7$ to $2.7-4.7$
228 $\times 10^6$ cells/ml in the FL₀ RAS (Fig. 7a) towards the end of the experiment (week 7). The number of free-living
229 bacteria in the FL_{3.13} RAS was stable ($0.8-1.2 \times 10^7$ cells/ml in week 7; Fig. 7, b), whereas in FL_{6.25} an increase
230 was observed over time (ending at $5.3-9.5 \times 10^7$ cells/ml; Fig. 7, c). The replicated RAS systems behaved fairly
231 similar, but deviations increased with increasing feed loadings.

232 3.3 Fish performance

233 Fish biomass in the FL_{3.13} and FL_{6.25} RAS increased by 14 and 28 kg/RAS, respectively during the experiment,
234 reaching 47 ± 0.49 and 61 ± 1.72 kg/RAS, respectively. The fish biomass in the FL₀ RAS was reduced by 0.4
235 kg/RAS. The FCR was 1.34 and 1.12 and SGR 0.48 and 0.81 % in the FL_{3.13} and FL_{6.25} RAS, respectively (FCR and
236 SGR in the FL₀ RAS not considered). Only limited mortality was observed over the 10 weeks experimental
237 period, ranging between 1 and 4 % of total biomass. The mortality was not different between the feed loading
238 regimes ($p < 0.05$).

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4. DISCUSSION

4.1 Physicochemical water quality assessment

The feed composition and feed loading applied and the concomitant TAN and urea excretion from the fish (Dalsgaard *et al.*, 2015) dictates the production of nitrate in RAS with well-functioning biofilters. Nitrate concentrations are hence predictable in RAS under steady state conditions, provided no removal of nitrate by denitrification (Colt *et al.*, 2006; Eding *et al.*, 2006; Pedersen *et al.*, 2012). Accordingly, three distinct scenarios in development of nitrate concentration were observed after the changes in feed loading (Fig. 2). The nitrate levels for the FL₀ RAS decreased exponentially towards 0 mg NO₃-N/L due to more-or-less ceased nitrate production and ongoing dilution. In the FL_{6.25} RAS nitrate increased towards twice the concentration of the control RAS (FL_{3.13}). The steady, minuscule increase in nitrate concentration in FL_{3.13} throughout the 10 weeks periods possibly reflect the slight increases in FCR (and increased TAN excretion) associated with fish getting larger, or it might be the asymptotic approach to complete balance.

The sudden increase in feed loading in FL_{6.25} did not cause any marked increase in TAN or nitrite concentrations, presumably due to the maturity of the system, the capacity of the biofilter applied and to substrate-dependent nitrification kinetics (Pedersen *et al.*, 2015; von Ahnen *et al.*, 2015). A doubling of ammonium loading is not a problem for a mature biofilm as long as it does not go into oxygen limitation (Harremoës and Henze, 1997).

The organic matter concentrations showed similar but less distinct patterns compared to the changes in nitrate concentrations. Before altered feed loadings BOD_{TOT} levels ranged between 5 and 10 mg O₂/L, even though all 6 RAS were kept under constant and identical conditions. This emphasizes the fact that identical RAS may differ substantially in some water quality parameters that actually affects bacterial communities. Whether such variation is unavoidable in biological systems or can be pinpointed to specific reasons cannot be determined from this study, but hydraulic conditions in the biofilter, uneven entrapment or liberation of particulate organic matter from the biofilter compartment (Fernandes *et al.*, 2017) and/or predation by protozoa and metazoa may affect systems specific carbon balances. Despite the initial variation, BOD_{TOT} in all systems diverged in accordance with changes in feed loading.

Likewise, the dissolved COD in FL₀ also decreased right after the feeding was ceased, while it remained relatively stable in the other two treatment groups. The decrease in COD_{DISS} in the FL₀ RAS was strongly correlated to the dilution. On the other hand, only a minor increase in COD_{DISS} was observed in the FL_{6.25} RAS, indicating a balance between production and removal of COD_{DISS} despite the loading.

The particulate fraction (COD_{PART}), however, did not respond in any linear or straightforward way to the feed loading because the largest increases and fluctuations were observed in the FL₀ RAS. This abrupt increment was not reflected in any BOD-fraction (data not shown), strongly suggesting that this transient increase in COD_{PART} was caused by biofilm release as a consequence of stopped feeding. For the FL_{3.13} and FL_{6.25} RAS, the COD_{PART} remained more stable, although some increase and also variation was observed between the FL_{3.13} systems at the end of the experiment. The COD_{DISS}/COD_{PART} ratio in FL_{3.13} at week 7 was 0.99, which is in accordance with Fernandes *et al.* (2015) who found a ratio of 0.93 after 19 weeks of operation under similar conditions. Probably more interesting, the difference between the FL groups was even more pronounced

when comparing BOD_{DISS}/BOD_{PART} . Ratios of 0.23, 1.14, and 4.14 were observed for FL₀, FL_{3.13} and FL_{6.25}, respectively, suggesting that this ratio could be used as a tool to indicate relative differences in feed loading and water quality between systems. The biodegradability index (BOD_{TOT}/COD_{TOT}) in the FL_{3.13} and FL_{6.25} RAS was approximately 0.09 at the end of the experiment, implying that the organic matter accumulating within the systems was > 90% recalcitrant (Rojas-Tirado *et al.*, 2017). In the FL₀ RAS the biodegradability index dropped to 0.06 at the end of the experiment, indicating a faster reduction in BOD than in COD, as could be expected.

The sludge collected from the fixed bed biofilters at the backwash event in week 7 was positively related to the feed loading level. The FL₀ RAS accumulated only 10% of the amount of sludge in the FL_{3.13} RAS. The FL_{6.25} RAS accumulated twice the sludge of the FL_{3.13} RAS. How deposition of particulate organic matter in fixed bed biofilters affects fluxes of dissolved and particulate organic matter as well as interactions between decomposers and grazers, deserves future attention.

4.2 Microbial water quality assessment

Bacterial activity

Bactiquant® levels were positively correlated to the changes in feed loading although a certain delay in response was observed. This is different from the immediate changes that occurred in nitrate concentrations. Bacterial activity in the FL₀ RAS did not decline after the feeding was stopped, but increased slightly towards the end of the experiment. Bactiquant® assesses bacterial activity by measuring a specific hydrolase enzyme found in most of bacteria, and Pedersen *et al.* (2017) demonstrated a linear correlation of Bactiquant® activity to the available surface area of particulate organic matter in less intense RAS water. The constant bacterial activity levels in FL₀ RAS suggests that the accumulation of particulate waste during the initial operation (before stopped feeding) was sufficient to sustain the bacterial growth on particles. This is supported by the fact that COD_{PART} actually increased in FL₀. The FL_{3.13} RAS - not subjected to changes - had increasing BQV in both RAS units, indicating that the systems at week 0 were not in steady state from a bacterial activity point of view. The Bactiquant®-response was related to the increases in organic matter levels observed (BOD_{TOT} and COD_{TOT}) in FL_{3.13} and in FL_{6.25} in particular. The BQV levels observed, range 2.7×10^4 - 1.5×10^5 BQV/ml, fit well to levels found in intensive RAS (Pedersen *et al.*, 2017). Bacterial activity in the FL_{6.25} systems, showed almost identical patterns between RAS duplicates, with a 3-4 weeks delay before a significant increase was observed. The increase might be expected as a result of the doubling in the feed load and the corresponding increase in waste excretion. However, apparently the biofilter was able to attenuate this change for a period of time.

Bacterial activity assessed by hydrogen peroxide degradation assay was significantly related to the feed loading ($p < 0.001$), supporting the hypothesis that feed loading directly dictates available organic matter influencing the microbial abundance and activity in RAS waters. Water from the FL₀ RAS had a significant removal of HP even 4-6 weeks after termination of feeding, suggesting a prolonged contribution of organic matter from e.g. biofilm release or from sludge, deposited in the biofilter. Arvin and Pedersen (2015) showed that HP degradation is a biotic process (no degradation of HP in autoclaved RAS water), related to microbial enzymatic activity rather than potential degradation due to inorganic catalysts (Pardieck *et al.*, 1992). The HP degradation assay applied turned out to be predictive and with sufficient reproducibility, pending to be implemented as a new, simple and fast method to evaluate bacterial water quality. Since HP degradation is

327 dependent on temperature and to a nominal concentration, assays have to be performed under similar
328 conditions in order to compare different water matrices and thus standard procedures has to be developed
329 for the method to be universally applied.

330 *Bacterial abundance of free-living cells*

331 Cell counting using flow cytometry have not been widely used to assess RAS microbial water quality but some
332 studies related to rearing of marine larvae in RAS have reported densities of $6-8 \times 10^6$ cell/ml and 1×10^7
333 cell/ml (Attramadal *et al.*, 2012, 2014), and 2×10^6 cell/ml (Wold *et al.*, 2014), 2×10^7 cell/ml in rearing water,
334 and $0.1-6 \times 10^6$ cell/ml in pure seawater inlet (van der Meeren *et al.*, 2011). Drinking water ranges between
335 10^3 to 10^6 cells/ml (Prest *et al.*, 2016a) and in this trial tap water used to fill the systems contained $0.5-1.4 \times$
336 10^6 cells/ml. These data may not be directly comparable to this study due to large differences in experimental
337 setups, especially feed loading. However, the cell numbers obtained in the present study are within the same
338 range.

339 The free-living cells in RAS water showed a direct response to changes in feed loading. The abundance in FL₀
340 RAS declined immediately after feed stop associated to dilution of the systems and the concomitant decline
341 in dissolved, readily available organic matter (BOD_{DISS}). However, a considerable amount of cells (of $2.7 - 4.7$
342 $\times 10^6$ cells/ml) were still measured at the end of the experiment for the FL₀ RAS. Although no distinction
343 between dead and living cells were made, the bacterial activity assessed by Bactiquant® supports the
344 presence of active cells. As discussed above, bacterial activity expressed by BQV may have been mainly
345 related to that bacteria attached to particles has higher extracellular enzymatic activity per cell than free-
346 living bacteria (Karner and Herndl, 1992; Smith *et al.*, 1995). Their activity could, together with microbial
347 degradation in the biofilter, have provided substrate for the free-living bacteria in the dissolved-substrate
348 limited water of the FL₀ RAS. This same interaction should, however, have been expected to happen in the
349 other treatments as well, but in a more dynamic way. The FL_{3.13} RAS showed a baseline of $0.5 - 4 \times 10^7$ cell/ml
350 between week 0 to week 7, ending with 60% more free-living bacteria than the FL₀ RAS. The FL_{6.25} RAS had a
351 comparable and simultaneous development with positive response and a pronounced increase in numbers
352 at the end of the trial, despite some difference in cell concentration between duplicates, exceeding the FL_{3.13}
353 RAS by five to nine times in cell number at the end of the experiment.

354 **4.3 Implications and challenges in RAS microbial water quality**

355 Increased feed loading caused direct but somewhat delayed responses in terms of bacterial activity and
356 abundance, implying that probably the biofilter attenuated the bacterial response in the water phase when
357 feed loading was increased. Mature biofilms shows rapid response to increased loading of inorganic and
358 organic nutrients to the system, and may also contribute to the dispersion of new bacterial cells into the
359 water (Leonard *et al.*, 2000; Davies, 2011; McDougald *et al.*, 2011). In this study, the results showed of an
360 almost immediate and constant increase in numbers of free-living bacteria in the FL_{6.25} RAS without
361 manipulating the C/N ratio (Leonard *et al.*, 2002; Michaud *et al.*, 2006). It seems that biologically mature
362 biofilter systems have the potential to assimilate a sudden increase in feed loading with concomitant increase
363 in attached and free-living bacteria. The difference in response between free-living bacteria and particle-
364 associated bacteria could have been related to the capacity of fixed bed biofilter for micro particles
365 entrapment (Fernandes *et al.*, 2017). As mentioned before, bactiquant is highly associated to bacteria

366 attached to particles, and the transition of water passing through the biofilter could have suppressed the
367 response in bacterial activity response in the water phase.

368 Heterotrophic and autotrophic bacteria will be in balance at system level as long the C/N ratio is not
369 dramatically changed by e.g. excess feed waste or insufficient solids removal (Fernandes *et al.*, 2015) and the
370 autotrophs not suffer from oxygen limitation. To evaluate such changes or to quantify effects of disinfection
371 (Attramadal *et al.*, 2014), new measures to detect bacterial abundance and activity including live/dead assays
372 are needed.

373 A number of well-known factors (e.g. organic and inorganic nutrients, temperature, pH, and predation)
374 regulate bacterial growth in water (Blancheton *et al.*, 2013; Gerardi, 2006; Rurangwa and Verdegem, 2015;
375 Prest *et al.*, 2016a), and more knowledge is needed to fully understand the interaction of these factors in
376 RAS. Monitoring tools like Bactiquant®, HP degradation assay, flow cytometry - used in this study, and others
377 like online flow cytometry (Besmer *et al.*, 2014), Bacmon (Grundfos A.S, Højris *et al.*, 2016) and ATPase assay
378 (Vang *et al.*, 2014) are all new measures that might provide means for an increased understanding of the
379 microbial dynamics within RAS. These approaches may all contribute to improving our understanding of the
380 complex microbial interactions in RAS in future studies. Additionally, surveys on full-scale RAS may also
381 increase our knowledge on how various factors affects the bacterial dynamics within a system that is
382 constantly challenged by variations in nutrient and organic loading.

383 5. CONCLUSION 384

385 This study demonstrated the dynamics in microbial water quality parameters as a function of increased and
386 decreased feed loading in a set of controlled pilot-scale RAS using two new fast and practical assays and flow
387 cytometry. The main conclusions are:

- 388 • Changes in feed loading caused substantial effects on selected chemical and microbial water quality
389 parameters. As the chemical response is immediate, microbial water quality response speed is
390 dependent on other influencing factors within RAS.
- 391 • Submerged biofilters attenuated the microbial response and fluctuations in microbial water quality
392 in RAS water when feed loading was increased.
- 393 • Bacterial activity measured as BQV or as HP degradation rate, responded to altered feed loading after
394 some weeks.
- 395 • Free-living bacteria responded to changes in the concentrations of dissolved organic matter.
- 396 • Bacterial activity and abundance within mature RAS were affected by substrate availability, solids
397 removal and particulate matter (surface area) and the submerged fixed-bed biofilter attenuated the
398 response observed in the water phase.

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577 **Figures and Tables**

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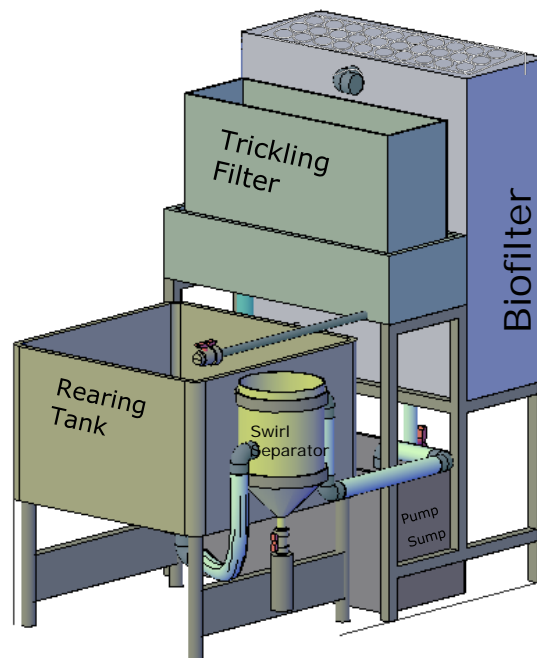


Fig. 1: Scheme of the RAS configuration, consisting of a fixed-bed biofilter (0.76 m³), trickling filter, rearing tank, swirl separator, and pump sump (more system details can be found in Pedersen *et al.*, 2012).

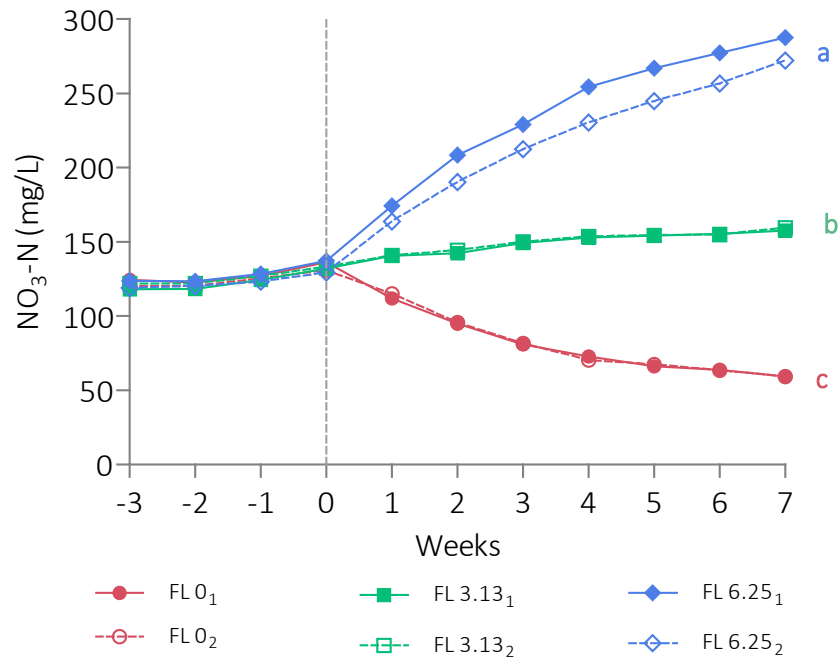
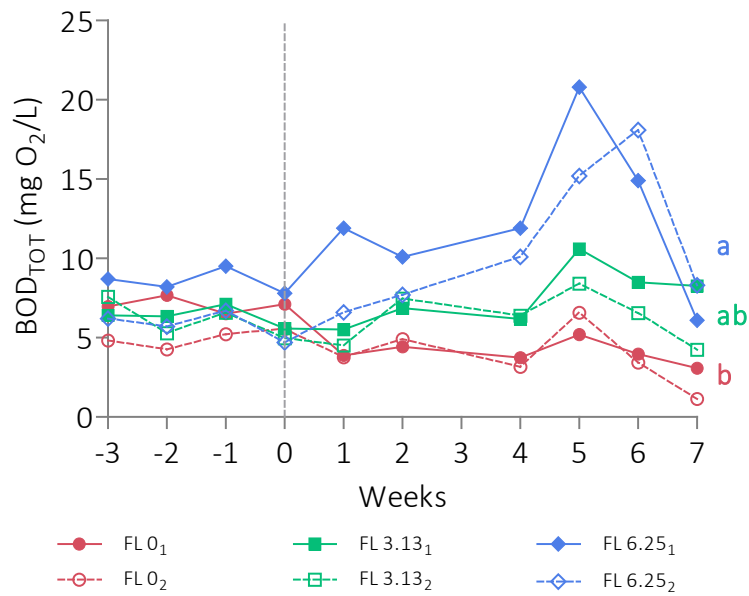


Fig. 2: Nitrate concentration over a period of 10 weeks for the different treatments and RAS duplicates: 0 kg feed/m³ (FL₀); 3.13 kg feed/m³ (FL_{3.13}); and 6.25 kg feed/m³ (FL_{6.25}). Week 0 and the vertical line indicates the last measurement before changes in feed loading were made. The minus weeks indicates concentration of nitrate in RAS water before changes in feed loading were made. Different superscript indicates statistical difference at week 7.



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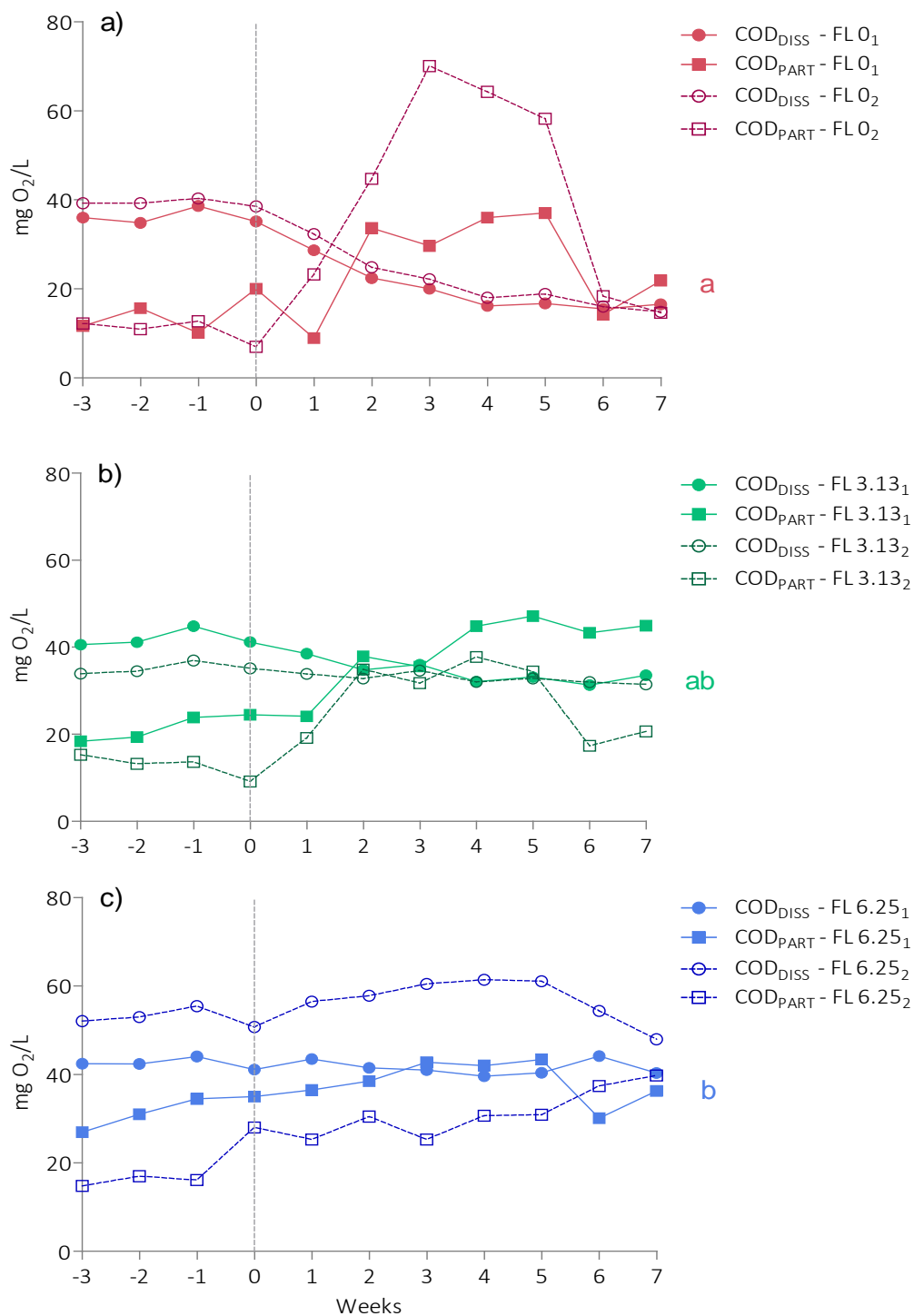
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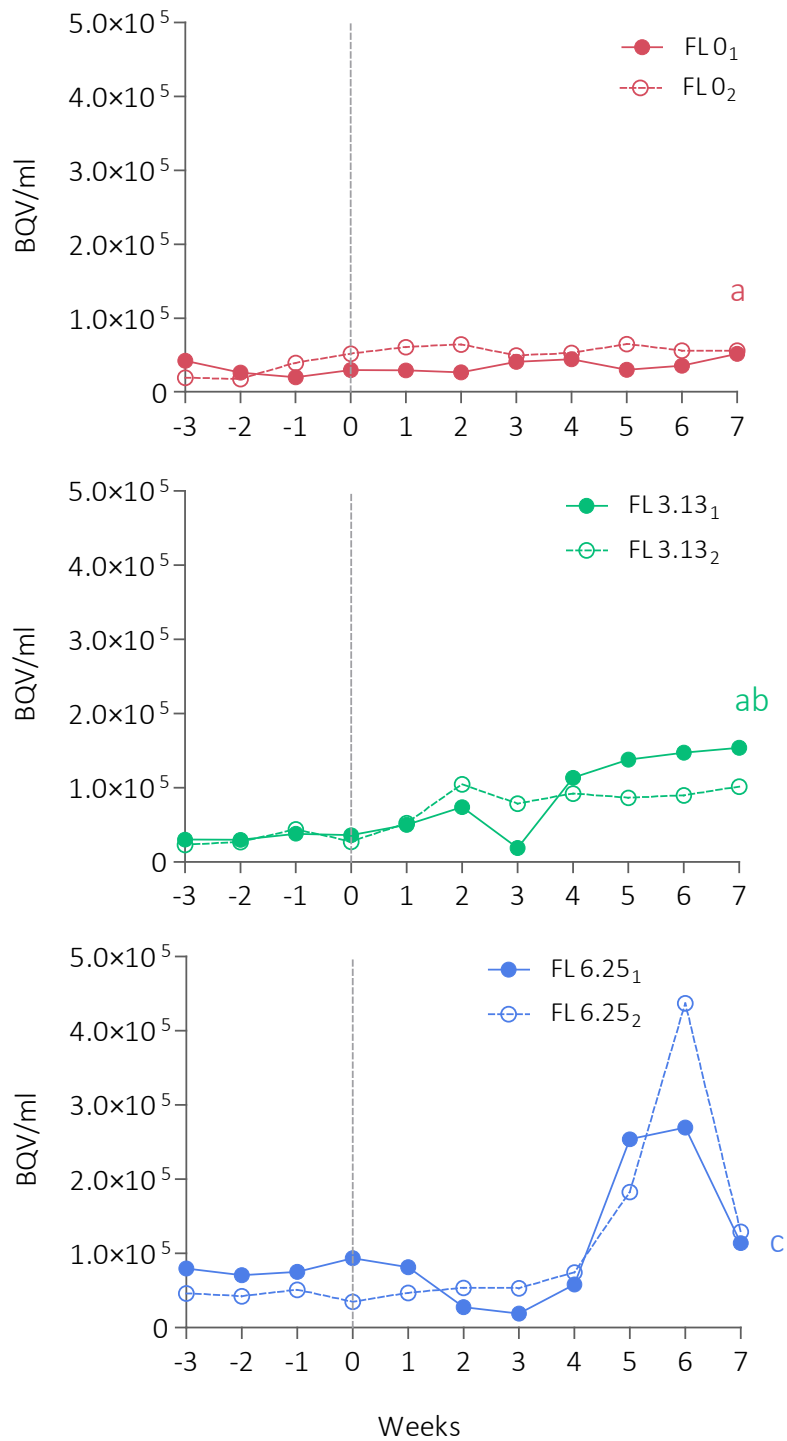
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Fig. 3: Total biological oxygen demand (BOD_{5-TOT}) concentration over a period of 10 weeks for the different treatments and RAS duplicates: 0 kg feed/m³ (FL₀); 3.13 kg feed/m³ (FL_{3.13}); and 6.25 kg feed/m³ (FL_{6.25}). Week 0 and the vertical line indicates the last measurement before changes in feed loading were made. The minus weeks indicates concentration of BOD_{TOT} in RAS water before changes in feed loading were made. Different superscript indicates statistical difference at week 7.



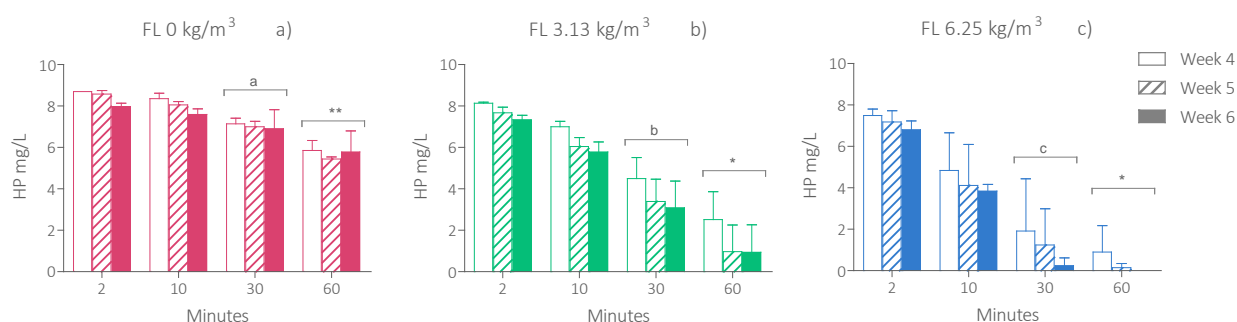
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613 **Fig. 4: Concentration of the dissolved and particulate fractions of COD over a period of 10 weeks for the different**
614 **treatments different treatments and RAS duplicates: 0 kg feed/m³ (FL₀); 3.13 kg feed/m³ (FL_{3.13}); and 6.25 kg feed/m³**
615 **(FL_{6.25}). Week 0 and the vertical line indicates the last measurement before changes in feed loading were made. The**
616 **minus weeks indicates concentration COD_{DISS} and COD_{PART} in RAS water before changes in feed loading were made.**
617 **Different superscript indicates statistical difference for COD_{DISS} between treatments at week 7.**



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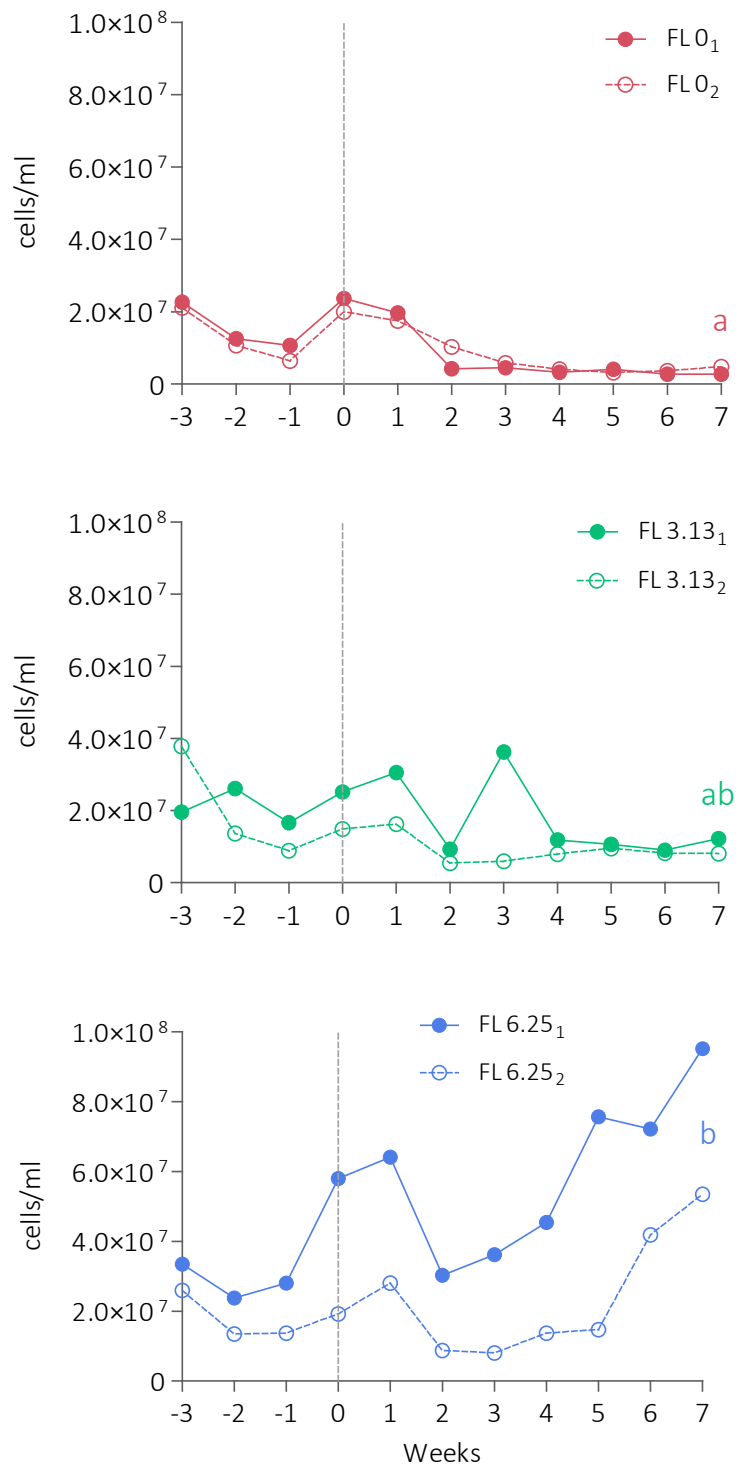
619 **Fig. 5: Bacterial activity measured as bactiquant values (BQV) over a period of 10 weeks for the different treatments**
620 **different treatments and RAS duplicates: 0 kg feed/m³ (FL₀); 3.13 kg feed/m³ (FL_{3.13}); and 6.25 kg feed/m³ (FL_{6.25}).**
621 **Week 0 and the vertical line indicates the last measurement before changes in feed loading were made. The minus**
622 **weeks indicates concentration of BQV in RAS water before changes in feed loading were made. Different superscript**
623 **indicates statistical difference between treatments at week 7.**



624

625 **Fig. 6: Hydrogen peroxide concentration (mean \pm SD, n=2) measured during 60 minutes in water samples from three**
 626 **different feed loadings (FL): a) FL 0 kg/m³; b) FL 3.13 kg/m³; and c) 6.25 kg/m³. Test was performed for water samples**
 627 **in week 4, 5 and 6. Blank bars: week 4; striped bars: week 5; and grey bars: week 6. Different superscript (30 min)**
 628 **and asterisk (60 min) indicates statistical difference between treatments (Tukey's test; $\alpha = 0.05$). Removal rate**
 629 **constants (k) achieved were (mean \pm SD, n=3): a) 0.41 ± 0.05 /h for FL 0 kg/m³; b) 1.73 ± 0.38 /h for FL 3.13 kg/m³; and**
 630 **c) 4.92 ± 0.86 /h for FL 6.25 kg/m³.**

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633 **Fig. 7: Concentration of free-living bacteria in RAS water from three different feed loading: a) 0 kg feed/m³ (FL₀); b)**
 634 **3.13 kg feed/m³ (FL_{3.13}); and c) 6.25 kg feed/m³ (FL_{6.25}). All graphs shown with time line of 10 weeks. Week 0 and the**
 635 **vertical line indicates the last measurement before changes in feed loading were made. The minus weeks indicates**
 636 **concentration of bacterial cells in RAS water before changes in feed loading were made. Different superscript**
 637 **indicates statistical difference between treatments (Tukey's test; $\alpha = 0.05$).**

638 **Table 1: Feeding load for maturation period and three treatment groups.**

	Adaptation period (Three months)	Treatment Groups		
		FL ₀	FL _{3.13}	FL _{6.25}
Feed quantity (g/d)	250	0	250	500
Water exchanged per day (m ³ /d)	0.08	0.08	0.08	0.08
Water renewal (m ³ /kg feed)	0.32	-	0.32	0.16
Feed loading (kg/m³)	3.13	0	3.13	6.25

639

640 **Table 2: Chemical water quality parameters and analytical methods applied.**

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical Method	Reference	Frequency of Measurement
Temperature, pH, dissolved Oxygen	Temp., pH, O ₂	°C, pH units, mg/L	Direct / on location	Hach HQ40d instrument, Hach Lange, Germany	N/A	Daily
Chemical oxygen demand	COD _{TOT}	mg O ₂ /L	Unfiltered + acid addition and kept at 4°C.	LCK 914, Hach Lange, Germany	N/A	Weekly
Dissolved chemical oxygen demand	COD _{DISS}	mg O ₂ /L	Filtered 0.22 µm and kept at 4°C.	LCK 914, Hach Lange, Germany	N/A	Weekly
Particulate chemical oxygen demand	COD _{PART}	mg O ₂ /L	N/A	COD _{PART} = COD _{TOT} - COD _{DISS}	N/A	Weekly
Biochemical oxygen demand of 5 days	BOD _{5-TOT}	mg O ₂ /L	Unfiltered	Potentiometry/O ₂ probe (WTW Oxi 340i)	ISO 5815	Weekly
Dissolved biochemical oxygen demand of 5 days	BOD _{5-DISS}	mg O ₂ /L	Filtered 1.6 µm	Potentiometry/O ₂ probe (WTW Oxi 340i)	ISO 5815	Weekly
Particulate biochemical oxygen demand of 5 days	BOD _{5-PART}	mg O ₂ /L	N/A	BOD _{5-PART} = BOD _{5-TOT} - BOD _{5-DISS}	N/A	Weekly
Total solids (dry matter) and ashes	-	g/L	Unfiltered	Gravimetric	NMKL.23	End of trial
Total ammonia nitrogen	TAN	mg/L	Filtered 0.22 µm. Conserved at 4°C.	Colorimetry	DS 224	Weekly
Nitrite	NO ₂ -N	mg/L	Filtered 0.22 µm. Conserved at 4°C.	Colorimetry	DS 223	Weekly
Nitrate	NO ₃ -N	mg/L	Filtered 0.22 µm. Conserved at 4°C.	Colorimetry	ISO 7890/1	Weekly

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642 **Table 3: Microbial water quality parameters and analytical methods applied.**

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical Method	Reference	Frequency of measurement
Bacterial Activity	BactiQuant Value	BQV	Unfiltered. Processed immediately	BactiQuant® (Mycometer, Denmark)	Manufacturers protocol	Weekly
HP degradation	HP	HP mg/L or h ⁻¹	Unfiltered. Processed immediately	Colorimetry	(Arvin and Pedersen, 2015)	Week 4, 5 and 6
Bacteria cell number	Cell number	cell/ml	Unfiltered. Fixed with glutaric aldehyde (1% final concentration). Freezed immediately with liquid nitrogen gas and conserved at - 20°C. Processed 6 months later.	Stained with Sybr Green I and counted with Flow Cytometer (Becton Dickinson FACscan)	(Marie <i>et al.</i> , 2005; Wold <i>et al.</i> , 2014)	Weekly

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Table 4: Water quality parameters before changes in feed loading were made (week 0) and at the end of experiment (week 7) for each treatment.

Parameters	Units	Week 0				Week 7			
		FL ₀ (n = 2)	FL _{3,13} (n = 2)	FL _{6,25} (n = 2)	FL ₀ (n = 2)	FL _{3,13} (n = 2)	FL _{6,25} (n = 2)	FL ₀ (n = 2)	FL _{3,13} (n = 2)
Dissolved Oxygen	mg/L	8.2 ± 0.01	9.1 ± 0.48	8.6 ± 1.21	8.3 ± 0.19	7.9 ± 0.68	6.9 ± 1.27		
pH		7.3 ± 0.04	7.4 ± 0.01	7.3 ± 0.03	7.3 ± 0.08	7.3 ± 0	7.4 ± 0.22		
Temperature	°C	19.4 ± 0.14	19.1 ± 0.21	19.7 ± 0.07	18.8 ± 0.07	19.1 ± 0.07	19 ± 0.07		
TAN	mg/L	0.3 ± 0.06	0.3 ± 0.08	0.3 ± 0.08	0.2 ± 0.07	0.4 ± 0.05	0.5 ± 0.03		
NO ₂ -N	mg/L	0.1 ± 0.02	0.1 ± 0	0.1 ± 0.01	0.1 ± 0.05	0.2 ± 0.02	0.2 ± 0.02		
NO ₃ -N	mg/L	134 ± 3.7	133 ± 0.9	133 ± 5.6	59 ± 0	159 ± 1.3	280 ± 11		
COD _{TOT}	mg O ₂ /L	50.4 ± 6.8	55 ± 15.1	77.5 ± 1.9	34 ± 6.3	65.3 ± 18.7	82 ± 7.8		
COD _{DISS}	mg O ₂ /L	36.9 ± 2.4	38.2 ± 4.3	45.9 ± 6.8	15.7 ± 1.2	32.5 ± 1.5	44 ± 5.4		
COD _{PART}	mg O ₂ /L	13.5 ± 9.24	16.8 ± 10.9	31.5 ± 4.9	18.2 ± 5.1	32.8 ± 17.2	38 ± 2.4		
BOD _{5-TOT}	mg O ₂ /L	6.3 ± 1.1	5.3 ± 0.4	6.3 ± 2.2	2.1 ± 1.4	6.3 ± 2.8	7.2 ± 1.6		
BOD _{5-DISS}	mg O ₂ /L	2.9 ± 0.2	3.2 ± 0.8	3.7 ± 0.04	0.4 ± 0.1	2 ± 0.8	5.8 ± 1.9		
BOD _{5-PART}	mg O ₂ /L	3.4 ± 1.3	2.1 ± 0.3	2.5 ± 2.3	1.7 ± 1.2	2.9 ± 3.6	1.4 ± 0.3		

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HIGHLIGHTS

[Rojas-Tirado *et al.*, “Changes in microbial water quality in RAS...”]

- Increased feed loading causes increase in bacterial activity and abundance in RAS water.
- Nitrate and organic matter showed an immediate response to feed loading changes in RAS water while microbial water quality parameters had an attenuated response.
- Bacterial activity in RAS water was assessed through hydrogen peroxide degradation assay – a novel, simple, rapid and culture-independent method.
- Microbial water quality monitoring in RAS water provide valuable information regarding system operational management.

1 **Monitoring abrupt changes in bacteria within biological stable RAS water**

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6
7 *Keywords: Water quality, bacterial activity, substrate, BactiQuant[®], recirculating aquaculture system (RAS),*
8 *acetate.*

10 **Abstract**

11 Several factors such as e.g. feed spill, insufficient solids removal, and moribund fish could lead to abruptly
12 and potentially changes in microbial water quality in recirculating aquaculture systems (RAS). There is a lack
13 of knowledge regarding the dynamic of changes in bacteria within RAS and on causal-relationships such as
14 e.g. biofilter bacterial population and bacterial population in the water phase. The use of reliable monitoring
15 tools to assess microbial water quality are required in order to achieve a better understanding of the different
16 processes within RAS.

17
18 This study evaluated how an abrupt increase in easily biodegradable carbon source affected bacterial activity
19 and abundance in a stable recirculating aquaculture system. The experiment was carried out in twelve
20 identical and separate RAS with a feed loading of 1.6 kg/m³ make-up water, operated during 4 months under
21 steady-state conditions to achieve stable conditions. Half of the RAS (n =6) were modified and left without
22 biofilter media. The twelve RAS were divided into four triplicated treatment groups defined as: i) control with
23 biofilter (c+b); ii) control without biofilter (c-b); iii) acetate with biofilter (a+b); and iv) acetate without
24 biofilter (a-b). The treatments a+b and a-b were spiked with 40 mg/L acetate three times at time 0 h, 24 h
25 and 48 h, where acetate degradation within the RAS was measured. Changes in bacterial activity was
26 assessed during 72 hours using BactiQuant[®], hydrogen peroxide (HP) degradation assay. Bacterial abundance
27 was assessed by counting micro-particles and flow cytometry.

28
29 The applied bacterial monitoring methods detected abrupt increments in activity and abundance of water
30 phase bacteria 24 hours after adding the acetate, followed by a rapid decay once acetate availability became
31 limited. It was also seen that in the systems having biofilter, the acetate was consumed primarily by bacteria
32 attached to the biofilter media.

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39 Introduction

40 The majority of the bacterial population in RAS water are heterotrophic (Leonard *et al.*, 2000; Michaud *et al.*,
41 2009; Michaud *et al.*, 2014; Rud *et al.*, 2017). Heterotrophic bacteria obtain their energy from degradation
42 of organic carbon compounds (Polanco *et al.*, 2000; Bitton, 2011). Of the elemental composition of bacteria
43 (carbon, nitrogen and phosphorus), carbon is often the growth limiting compound within RAS (Leonard *et al.*,
44 2000). Therefore, additional biodegradable organic matter within RAS is expected to cause an increase in
45 heterotrophic bacteria. According to literature, a dissolved carbon concentration of 1 µg C/L is enough to
46 promote the growth $10^3 - 10^4$ cells/ml (Prest *et al.* 2016).

47 Carbon to nitrogen ratio close to 1 has been suggested to promote a stable development between
48 autotrophic and heterotrophic bacterial population within biofilters (Avnimelech, 1999, Zhu and Chen, 2001,
49 Nogueira *et al.*, 2002, Michaud *et al.*, 2006). This ratio is dependent on feed composition, feed digestibility
50 (Michaud *et al.*, 2014) and the efficiency of the organic load removal treatments (Fernandes *et al.*, 2015).
51 However, C/N ratio affect microbial growth in the water phase to a less extent, as most of ammonium is
52 degraded by the nitrifiers in the biofilter. Suspended growth in the water phase is mostly related to
53 heterotrophic growth that is regulated by the availability of the carbon source referred to as carrying
54 capacity.

55 The carrying capacity (CC) is defined as the amount of organic matter that can sustain a number of microbes
56 within a system over time (Vadstein, 1993). The CC within a RAS is in general, dictated by the amount of
57 substrate availability for bacteria to grow (Attramadal *et al.*, 2012). Under normal operational conditions, the
58 CC can fluctuate due to altered feed input, therefore bacteria within the systems will grow and be regulated
59 accordingly to this CC level. There are several factors such as e.g. feed spill, insufficient solids removal , and
60 moribund fish which could increase the CC abruptly and potentially cause changes in microbial water quality
61 in RAS in terms of abundancy and diversity (Attramadal *et al.* 2012, 2016; Wold *et al.*, 2014).

62 Biofilters in RAS ensure removal of ammonia and affect organic matter turnover by retaining (case of fixed
63 bed filter), releasing (moving bed) and degrading organic matter (Rusten *et al.*, 2006; Fernandes *et al.*, 2017).
64 Some studies have shown that the abundance of free-living bacteria in RAS rearing water have a direct
65 correlation to the abundance of attached bacteria to biofilter media (Leonard *et al.*, 2000; Michaud *et al.*,
66 2006). In a previous study (Rojas-Tirado *et al.*, in review) it was presumed that the biofilter may have
67 attenuated the immediate response of bacterial in the water phase for when the feed loading was increased.
68 As a well-known feature of biofilters, mature bacterial community in the biofilter consumes the extra
69 available carbon source fast (Davies, 2011), limiting the available carbon for bacteria further down in the
70 distribution and thus, regulate the microbial water quality. Monitoring tools may be able to reveal
71 fundamental mechanisms as causal-relationships, which needs to be elucidated for optimizing monitoring
72 strategies and selection of proper monitoring tools.

73 This study was made to gain increased understanding on bacterial response in RAS water when additional
74 organic load, simulating e.g. feed spill in a tank occur. Addition of acetate as an easy biodegradable carbon
75 source was applied to previously conditioned RAS (biological stable) as daily pulse for three consecutive days.
76 Bacterial abundance and activity in the water phase were assessed with a set of monitoring tools and the
77 suppression effect of the biofilter on microbial water quality was also evaluated.

78 2. Material and Method

79 2.1 System maturation

80 The experimental trial took place at the facilities of DTU Aqua in Hirtshals, Denmark. Twelve identical pilot
81 freshwater RAS 1.7 m³ were used for the trial. The technical setup of the RAS is described in Pedersen et al,
82 (2012), with one modification that consisted on the removal of half of the biofilter media to match the
83 feeding capacity. Each system was stocked with 12.5 Kg of rainbow trout (*Oncorhynchus mykiss*) and fed with
84 125 g feed/day. The daily water exchange for each RAS was 80 L/day. Systems maintenance took place every
85 day at 9:00 to 10:00, including removal of solids by emptying the accumulated feces in the collector below
86 each swirl separators, water exchange, and addition of bicarbonate to compensate alkalinity loss due to
87 nitrification, and measurements of water quality parameters. All systems were operated for five months
88 (from December to April) to ensure biological and physical-chemical stable water.

89 2.2 Experimental procedures

90 Two experiments were carried out in order to: 1) document the effect acetate – as a easy biodegradable
91 substrate) had on bacterial abundance and activity in RAS water simulating e.g. feed spill; and 2) evaluate
92 the suppression effect of the biofilter upon bacteria in RAS water after addition of easy biodegradable
93 substrate.

94 2.2.1 EXPERIMENT 1 (batch experiment)

95 To test the effect of easy biodegradable on bacterial abundance and activity in RAS water, a beaker trial was
96 carried out. Acetate was chosen as the easy biodegradable carbon source and was added in three different
97 concentration (Table 1): low concentration (LC) of 10 mg acetate/L, middle concentration (MC) of 20 mg
98 acetate/L, and a high concentration (HC) of 40 mg acetate/L.

99 The RAS water was taken from a random tank. It was carefully homogenized and distributed into eight 2 L
100 beakers. The water in the beakers was spiked further with the different concentration of acetate (Table 1)
101 and each concentration was tested in duplicates. Two beakers were kept as control. All beakers were
102 supplied with sufficient aeration and were stirred at a velocity of 100 RPM to keep sample homogenized. The
103 experiment was run at fixed temperature of 17 °C, simulating the same temperature of the RAS water.
104 Evaluation period was 72 h.

105 2.2.2 EXPERIMENT 2 (pilot-scale RAS)

106 To test and evaluate biofilter effect on acetate residual, twelve previously conditioned RAS were used. Six of
107 these RAS got their biofilter media removed 5 hours before the start of the trial in order to let the system
108 stabilize, and the other six RAS kept the biofilter. Four treatment groups were then established in triplicate
109 RAS. The treatments were the following: i) control RAS with biofilter (c+b); ii) control RAS without biofilter
110 (c-b); iii) RAS spiked with acetate with biofilter (a+b); and iv) RAS spiked with acetate without biofilter (a-b).
111 Each RAS from group (a+b) and (a-b) were spiked with 40 mg acetate/L at time zero and after 24 and 48
112 hours. The total evaluation period was 72 h.

113

114 2.4 Water sampling and analysis

115 Water grab samples were taken in a standardized way (time, volume, location and person). Each sample was
116 siphoned gently from the pump sump of each RAS before the start of daily routine processes. Water samples
117 were analyzed for bacterial activity and abundance, and ammonium and nitrite concentration were
118 measured to assess performance of biofilter. Table 2 lists sampling procedure, treatment and processing of
119 each parameter for microbial and physicochemical water quality. For Experiment 1, water samples were
120 samples were taken at time zero, after 6, 24, 48 and 72 hours from each beaker. For Experiment 2, water
121 samples were taken at time zero and every 2, 6, 12, 24, 30, 48, 54 and 72 hours from each RAS.

122 Bacterial activity in the water was assessed with Bactiquant® method and hydrogen peroxide (HP).
123 Bactiquant® measures bacterial activity indirectly via a common hydrolase enzyme found within a wide range
124 of bacteria (Reeslev *et al.*, 2011). A well-defined water volume was filtered through a 0.22 µm filter, on which
125 particle-bound and free bacteria are trapped; the filter cake is then exposed to a fluorescent substrate and
126 depending on the amount of bacteria present and their activity, a quantitative fluorescent signal can be
127 detected. The BQ values (BQV) were calculated according the sample volume (10 ml), exposure time (30 min)
128 and incubation temperature (measured on site) as described by Rojas-Tirado *et al.*, (2017) and Pedersen *et*
129 *al.* (2017). Bactiquant was measured at time zero, after 6, 24, 48 and 72 hours in Experiment 1 whereas for
130 the Experiment 2, it was only measured in the beginning (time= 0 h) and at the end of the trial (time = 72 h).

131 Hydrogen peroxide degradation assay applied was based on the microbial activity degradation kinetics as
132 described in Arvin and Pedersen (2015). Water samples were taken from the pump sump of each RAS and
133 transferred to 50 ml vials. The vials were put on a shaking water bath (Julabo SW 22) at 100 RPM and at 22
134 °C. The vials were spiked with HP to reach a nominal HP concentration of 8 mg H₂O₂/l. Hydrogen peroxide
135 concentrations were measured 0.5, 15, 45 and 60 minutes after HP addition to quantify degradation rates.
136 Hydrogen peroxide residual was measured spectrophotometrically according to Tanner and Wong (1998)
137 and Pedersen and Pedersen (2012). Data for the HP removal in this study are presented by the exponential
138 reaction rate (k) calculated from the exponential decay equation: $C_t = C_0 * e^{-kt}$, expressed as h⁻¹.

139 Micro-particles ranging between 1 to 30 µm in diameter were counted with a coulter counter (Beckman
140 coulter, Multisizer 4e) . The total number of suspended bacteria cells were quantified by flow cytometry
141 (Becton Dickinson FACscan) using SYBR Green II (RNA gel stain in DMSO) as the fluorescent dye. Bacteria cells
142 abundance was determined by gating on the FL1-versus-FSC plot shown in Fig.1. For this trial, the water
143 samples did not receive any treatment that could provide cell detachment from particles. Therefore, in this
144 sense, the data obtained during this trial are referred to bacteria cells suspended in the water phase or also
145 called “free-living bacteria”. Free-living cells were only counted for the 12 RAS trial with and without
146 biofilters.

147 The total ammonia nitrogen (TAN) and nitrite-N were measured in the treatment RAS at the end of the
148 experiment for assessing effect on nitrification process due to the removal of the biofilter media and acetate
149 addition.

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152 2.4 Data Analysis

153 Data were normalized (C/C_0) in order to compare changes between the different parameters exposed to the
154 same treatments. To evaluate the effect biofilter had on the different parameters, each group (control group
155 with and without biofilter and the group spiked with acetate with and biofilter) were analyzed by a one-way
156 analysis of variance (ANOVA) and a probability level of 0.05 was used.

157

158 3. Results

159 3.1 EXPERIMENT 1 (batch experiment)

160 3.1.1 Acetate fate in RAS water

161 The unexposed RAS water samples had a dissolved COD (COD_{DISS}) of 35.55 mg O_2/L . After spiking with the
162 different concentration of acetate, the COD_{DISS} concentrations measured after 6 h were: 34.97, 42.25, 50.95
163 and 73.8 mg O_2/L for the control, low (LC), medium (MC) and high (HC) concentrations respectively. Acetate
164 at low and medium concentration (10 mg/L and 20 mg/L) was removed completely after 24 hours, whereas
165 the higher concentration (40 mg/L) was totally removed within 48 hours (Fig. 2).

166

167 3.1.2 Bacterial activity and micro particles in RAS water

168 Micro particles showed an equal increase in all beakers spiked with organic carbon, until 24 h were they
169 deviated from each other accordingly to the amount of the organic load provided. The final values obtained
170 ranged from 1.8 to 3.3×10^6 particles/ml for the LC to the HC (Fig. 3, c; Table 3). The control decreased
171 constantly until the end of the trial from 2 to 1.2×10^6 (Fig. 3, a; Table 3).

172 The rate constant (k) of HP degradation increased after 24 h, indicating increase in bacterial activity
173 consistently with the organic load concentrations. Thereafter, bacterial activity started to decrease, ending
174 with a significant removal rate constant (k) for the highest acetate concentration of $0.8\ h^{-1}$ compared to the
175 control, LC and MC (0.5 , 0.6 , $0.7\ h^{-1}$) respectively (Fig. 3, b; Table 3).

176 The bactiquant method (Fig. 3, c) showed no significant change between the different treatments and control
177 ($p > 0.05$), ranging in total from approximately 7 to 9×10^4 BQV/ml from start to end of trial (Table 3).

178 3.1.3 Effect on micro particles

179 Micro particles in the size range from 1 to $3\ \mu m$ contributed a 95% of the total number of particles originally
180 measured within the range of 1 to $30\ \mu m$. Figure 4 (a) shows the changes of particles between 1 to $3\ \mu m$
181 accordingly to the different acetate concentrations after 72 h exposure. In the control beakers, the highest
182 number of particles was within the size range of $1\ \mu m$, whereas in the LC, the highest peaks were found
183 between size range of 1 to approximately $1.2\ \mu m$. In the MC, particles in the size range of approximately $1.3\ \mu m$
184 had the highest peak and around $1.8\ \mu m$ for the HC. In Fig. 4 (b), the development of micro particles over
185 time for the control treatment are shown. No evident changes was observed over time. The development of

186 micro particles over time for one of the water spiked with high concentration of acetate (HC) is depicted in
187 Fig. 4 (c). Here, development of particle size after 24 h until the end of the trial (72 h) was observed. Water
188 samples from this treatment were put under the microscope and it was observed that the increase in size of
189 the micro particles where due to bacterial cell division. Formation of diplococci and tetrad where observed
190 (pers. obs).

191

192 **3.2 EXPERIMENT 2 (pilot-scale RAS)**

193 **3.2.1 Acetate degradation**

194 Following acetate spikes degradation was observed in RAS, both with and without biofilter (Fig. 5). All RAS
195 with biofilter (A+b) removed 76% of the acetate within 24 hours after the first acetate spike. Complete
196 acetate degradation was found after the second and third spike.

197 In the RAS without biofilter (A-b), after the third spike, 67 % of acetate was removed within 6 h (Fig 5, a). In
198 two of the RAS, 100% degradation of acetate happen within the 24 h. During the second spike, one of the
199 RAS (RAS K) removed the acetate at a 100 % after 6 h, followed by RAS J with a 67 %. Finally, after the third
200 spike all RAS removed from 80 to 100 % after 6 h.

201 **3.2.2 Bacterial activity**

202 Bacterial activity assessed with bactiquant method, ranged between $0.3 - 1.8 \times 10^5$ BQV/ml at the startup of
203 the trial within the different RAS (Table 4). After three days with consecutive addition of acetate, most of RAS
204 ended with BQV within the range of $0.2 - 3.3 \times 10^5 \text{ ml}^{-1}$, where one the RAS achieved a higher value (Table
205 4). Figure 7 shows the average of normalized data of BQV for the different treatments. The control group
206 with and without biofilter, almost all RAS ended with a small increase in BQV at the end of the trial, whereas
207 the RAS (a+b) achieved the lowest BQV. The treatment group (a+b) ended with a higher bacterial activity but
208 with larger variation (Fig. 6, Table 5).

209 Initial and final values of bacterial activity assessed with HP degradation assay can be found in Table 4 and 5
210 for the control and acetate groups. For both control groups with and without biofilter, the bacterial activity
211 assessed with HP degradation assay remained stable in most of the RAS during the three days of trial (Fig. 7;
212 a, b). The RAS (a+b) also revealed to be stable over time after consecutive acetate spike (Fig. 7, c). The
213 maximum increase in bacterial activity observed within the RAS group (a-b). After the second spike, a 10
214 times fold increase was observed in one of the RAS ($k = 3 \text{ h}^{-1}$). After the third spike at 48 h of trial, the increase
215 of HP removal rate was remarkably higher within this group (4.6 to 6.2 times increase) compared to the
216 control and the RAS group (a+b). At the end of the trial, bacterial activity within group (a-b) dropped to values
217 found at the beginning of the trial in two of the RAS (Table 5). The presence of biofilter had a significant effect
218 ($p > 0.05$) on bacterial activity in the water phase when comparing both groups with and without biofilter
219 spiked with acetate.

220

221

222 3.2.3 Bacterial abundance

223 The concentration free-living bacteria within both control groups (c-b and c+b) remained stable over the
224 three days of trial (Fig. 8; a, b). The RAS group (a+b) had a slighter more variation in free-living cells between
225 RAS and the time laps but never reached more than a two-time fold increase. The RAS without biofilter (a-b)
226 and spiked with acetate showed a constant increase up to a five-time fold in free-living bacteria after the
227 second spike. After the third spike bacteria in the RAS water started to decline. The results shows that when
228 comparing the two groups of RAS (a+b and a-b) spiked with acetate, the biofilter had a significant effect ($p <$
229 0.05) on free-living cells concentration in the water phase. The start-up and final bacteria cell concentrations
230 values are found in Table 4 and 5.

231 3.2.4 Micro particles

232 Micro particle numbers within the triplicate control RAS groups (c+b and c-b) remained stable over the trial
233 period with a maximum increase of 1.85 compared to the initial values in a RAS with biofilter (Fig. 9,a; Table
234 4). The presence or absence of biofilter had no effect ($p > 0.05$) on micro particle changes in the control
235 group.

236 The micro particles in the a+b RAS (Fig. 9, c), increased a 2.5 times fold after second spike in one of the RAS,
237 corresponding to 2.9×10^6 particles/ml. For the (a-b) RAS, 24 hours of the first spike the particle number
238 increased 6-7 times fold in two of the RAS. After the second spike, an increase up to 15 time-fold increase is
239 achieved followed by the 16 time-fold increase after the third spike. Biofilter showed to have a significant
240 effect ($p < 0.05$) on micro particles changes in RAS water. The start-up and final particle concentrations values
241 are found in Table 4 and 5.

242 3.2.5 Assessment of TAN and nitrite levels

243 The results for TAN concentration were 0.82 ± 0.08 mg TAN/L for the controls with and without biofilter (C+b
244 and C-b), and 1.08 ± 0.007 for the RAS spiked with acetate with or without biofilter (A+b and A-b). The nitrite-
245 N concentrations were 1.05 ± 0.03 mg $\text{NO}_2\text{-N/L}$ for the C+b and C-b treatments and 1.07 ± 0.003 mg $\text{NO}_2\text{-N/L}$
246 for the A+b and A-b treatment RAS (Mean \pm SD, $n = 6$).

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255 4. Discussion

256 Feed spill or insufficient solid removal can lead to sudden increase of the organic load in RAS water, increasing
257 the carrying capacity and thereby trigger growth of opportunistic bacteria. In this study, we evaluated the
258 influence in bacteria abundance and activity of mature RAS water after the abrupt addition of an easy
259 biodegradable organic source. In addition, the causal-relationship between biofilters bacterial population
260 and bacterial population in the water phase after pulse addition of an easy biodegradable substrate was also
261 addressed.

262

263 4.2 Experiment 1 (batch experiment)

264 Effect of bacteria in the water phase after acetate addition

265 Bacterial activity in the static water sample, measured with the HP degradation assay responded consistently
266 to the increase in organic loading. HP degradation assay, have catalase as enzyme target (Pardieck *et al.*,
267 1992; Arvin and Pedersen, 2015). Catalase is an enzyme found in most aerobic bacteria and it is used as a
268 defense mechanism when the cell is in contact with HP (Mishra and Imlay, 2012). Therefore, the increase of
269 HP degradation constant rate (k) indicated an increase in bacterial activity, related to microbial growth after
270 acetate addition.

271 The increase in micro particles numbers between 1 and 3 μm revealed bacterial growth. It was also observed,
272 that particles ranging between 1 to 3 μm corresponded to 95% of the total particles counted within a size
273 distribution of 1 to 30 μm . Changes in micro particles/bacteria were highly consistent with changes in
274 bacterial activity and the acetate concentrations supplied.

275 Addition of acetate into the water did not cause any effects on Bactiquant®. An explanation to this is that
276 Bactiquant® measures bacterial activity indirectly by targeting an enzyme belonging to hydrolase class
277 (Reeslev *et al.*, 2011). In the presence of acetate – which is a readily transportable and utilizable molecule of
278 low weight (Chróst, 1991; Canelhas *et al.*, 2017) - bacteria do not require the additional activation of
279 hydrolase due to the uptake of readily biodegradable substrate is by simple diffusion. Therefore, bacterial
280 activity assessed by Bactiquant® was constant even when different concentrations of substrate were added.
281 This observation may indicate that *de facto* changes in bacterial activity can be underestimated if only
282 assessed by the Bactiquant® method.

283 The no change in bactiquant and decrease in bacterial activity and micro particles in the controls beakers,
284 confirms that the RAS water was substrate limited. However, by adding additional organic load, the carrying
285 capacity (Attramadal *et al.*, 2012) in the water was expanded and thereby the growth of heterotrophic
286 bacteria was triggered as well.

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289

290 4.1 Experiment 2 (pilot-scale RAS)

291 Control RAS (c+b and c-b)

292 Both control treatment RAS with and without biofilter, maintained stable in terms of bacterial abundance
293 and activity and micro-particles over the experimental period. This implicate that the systems where
294 biological stable and that the removal of the biofilter media did not interfere with this stabilization in terms
295 of bacterial abundance and activity.

296 RAS with biofilter spiked with acetate (a+b)

297 The three RAS group with biofilter that were spiked with acetate showed, that the presence of the biofilter
298 suppressed proliferation of bacteria in the water phase, in terms of both activity and abundance. Despite the
299 amount of organic carbon source provided, simulating a $\times 6.7$ fold addition of feed on daily basis, the mature
300 community within the biofilter managed to reduce most of it. This feature of mature biofilm is well known
301 (Davies, 2011). In a previous study (Rojas-Tirado *et al.*, in review), it was presumed that the biofilter was
302 responsible for reducing the response in bacterial abundance and activity in the water column after feed
303 loading was increased. The results of the present study also confirms that biofilters have an effect on the
304 suspended bacteria further in the recycling loop and it is due to the substrate consumption in the biofilter.

305 Biofilter performance was not affected by the increase on the C/N ratio between these three days of trials.
306 Studies based on looking at C/N effects, have shown effect on nitrification rates operating at elevated C/N
307 only after several weeks (Michaud *et al.*, 2006; Zhu and Chen, 2001).

308 RAS without biofilter spiked with acetate (a-b)

309 The acetate consumption within the RAS without biofilter was very fast compared to the RAS with biofilter.
310 Within this treatment group, bacterial abundance and activity revealed distinct changes associated to the
311 easy biodegradable carbon source added.

312
313 Bacterial activity assessed with HP degradation assay had up to a 10 times fold increase after the second
314 spike of acetate (time = 30 h), ending all three RAS with 4 times fold increase after the third spike (54 h).
315 Bacterial activity measured with Bactiquant® within the (a-b) treatment group indicated an average 4 –fold
316 increase compared to the other treatment groups. However, this increase was mainly due to the high
317 increase in only one of the RAS. The same effect as in the HP degradation assay was observed for bacterial
318 abundances measured with flow cytometry. After the third spike a 4 fold increase in cells/ml was obtained
319 in the treatment group (a-b).

320
321 Bacteria in a RAS operating under constant conditions manage to stabilize accordingly to the carrying capacity
322 of the system after a period dependent on the residence time of the water within the system and the feed
323 load proportionated. Therefore, bacteria surviving within the systems are submitted to substrate-limited
324 conditions where the carbon source is the limiting source (Attramadal *et al.*, 2012; Leonard *et al.*, 2002; Rojas-
325 Tirado *et al.*, in review). It can then be hypothesized, that the low substrate input, is enough to uphold cellular
326 maintenance and therefore growth yield is low and stable. By adding acetate as pulse, the sudden and high
327 concentration/availability of substrate for bacteria in the water allowed bacteria cells to spend less energy
328 on transport or passive diffusion into the cell. Then, the sudden high amount of substrate may have allowed

329 bacteria in the water to allocate their metabolically energy on biomass production rather than for cellular
330 maintenance (Canelhas *et al.*, 2017) and thereby growth is triggered as shown by the monitoring tools used.

331 Micro particle measurements – which was associated with bacterial growth – had a clearer reaction to the
332 pulse additions of acetate. Here, changes in particles reached up to 15 to 16 times fold increase after the
333 second and third spike within the same treatment group. Though, the dynamic observed in micro
334 particles/bacteria was that cell number increased after each spike but also decreased 24 h after the spike. A
335 possible answer to this was related to the limitations of the instrument. Although the coulter counter method
336 counts numbers of cells, it also provides with information regarding cell size. However, the limitation for
337 when counting bacteria is due to their size range which is 0.2 to 3 μm (Gerardi, 2006). This means that
338 bacteria in the water phase may have increased in size for then to divide into new single and smaller cells,
339 which cannot be detected by the coulter counter.

340 According to literature, old bacterial cells belonging to the same species are bigger than the young cells and
341 have a higher growth rate. These new cells increase in size only to reproduce. The young bacterial cells have
342 an advantage of their larger surface-to-volume ratio compared to older bacterial cells. Higher surface-to-
343 volume ratio provides larger surface area for the absorption of substrate, and thereby a higher metabolic
344 rate of activity (Gerardi, 2006, Pedersen *et al.*, 2017). This explains the dynamic of the fast response on
345 bacterial growth in conditioned/biological stable RAS water.

346 Flow cytometry have the advantage compared to the coulter counter that it can differentiate bacteria from
347 inert particles by use of staining procedures. In addition, flow cytometry can be calibrated to count bacterial
348 within a smaller range (REF). However, the flow cytometer do not quantify size groups as the coulter counter.
349 Therefore, the combination of both of these methods can contribute for further understanding on bacterial
350 dynamic.

351 During the experimental period (three days), TAN and nitrite did not increase in the water. We assumed that
352 this was a consequence of the experimental setup applied, where the empty biofilter compartments may
353 have had tank surface attached bacteria or settled bacteria with nitrifying abilities similar to activated sludge
354 (Pedersen *et al.*, 2012)

355 The findings in this study illustrate the potential effects and some mechanisms of the addition of extra
356 substrate – here simulating feed spill or insufficient solid removal in RAS. It emphasizes the rapid implications
357 of changes on the bacterial dynamic in the water and probably also in the biofilm.

358 This study also revealed the complexity of water phase bacterial interactions (planktonic and particle
359 associated bacteria) with biofilm fixed bacteria. The bacterial growth and activity dynamics in a water
360 samples have recently been found to be significantly affected by the mode of substrate addition over a four
361 weeks period (Canelhas *et al.*, 2017) which should considered in future studies.

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366 5. Conclusions

367

- 368 i) Bacteria in the water phase of a stabilized/mature system will increase accordingly to the
369 amount of additional carbon source provided.
- 370 ii) Addition of extra easy biodegradable carbon source three times consecutive on a daily basis
371 did not show an increase in bacteria in the water phase compared to water from systems
372 where the biofilters were removed. This confirmed the biofilter great buffer capacity and
373 thereby the delay in the response of the microbial water quality parameters.
- 374 iii) The monitoring tools used for this purpose was Bactiquant®, FCM, HP degradation assay, and
375 particle coulter counter. All of them were able to detect the abrupt changes within microbial
376 water quality, except Bactiquant®. Here, Bactiquant® was insufficient to assess microbial
377 activity, which was attributed to the substrate added and the principle of the method itself.

378

379 6. References

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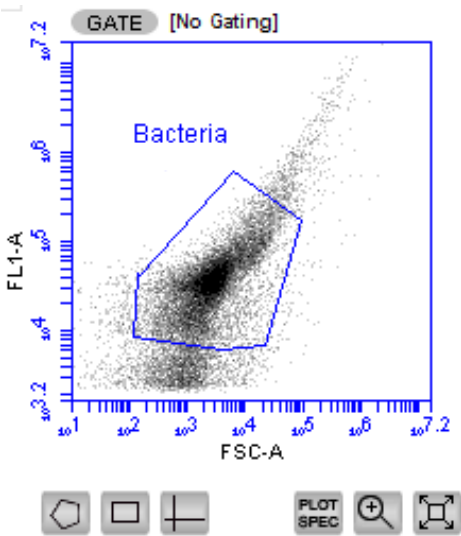
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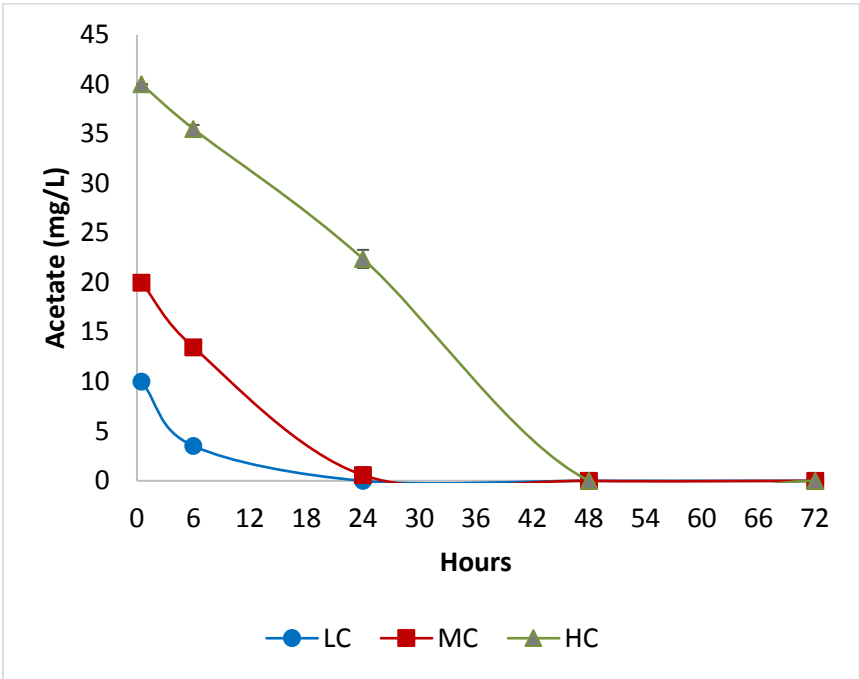


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489 **Fig. 1: Flow cytometry analysis of RAS water samples. Bacterial cells were stained with SYBR II. Cells were delimited**
490 **by gating with program BD Accuri™ C6 Software.**

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494 **Fig. 2: Degradation of the different acetate concentrations (10, 20 and 40 mg/L) over a period of 72 hours.**

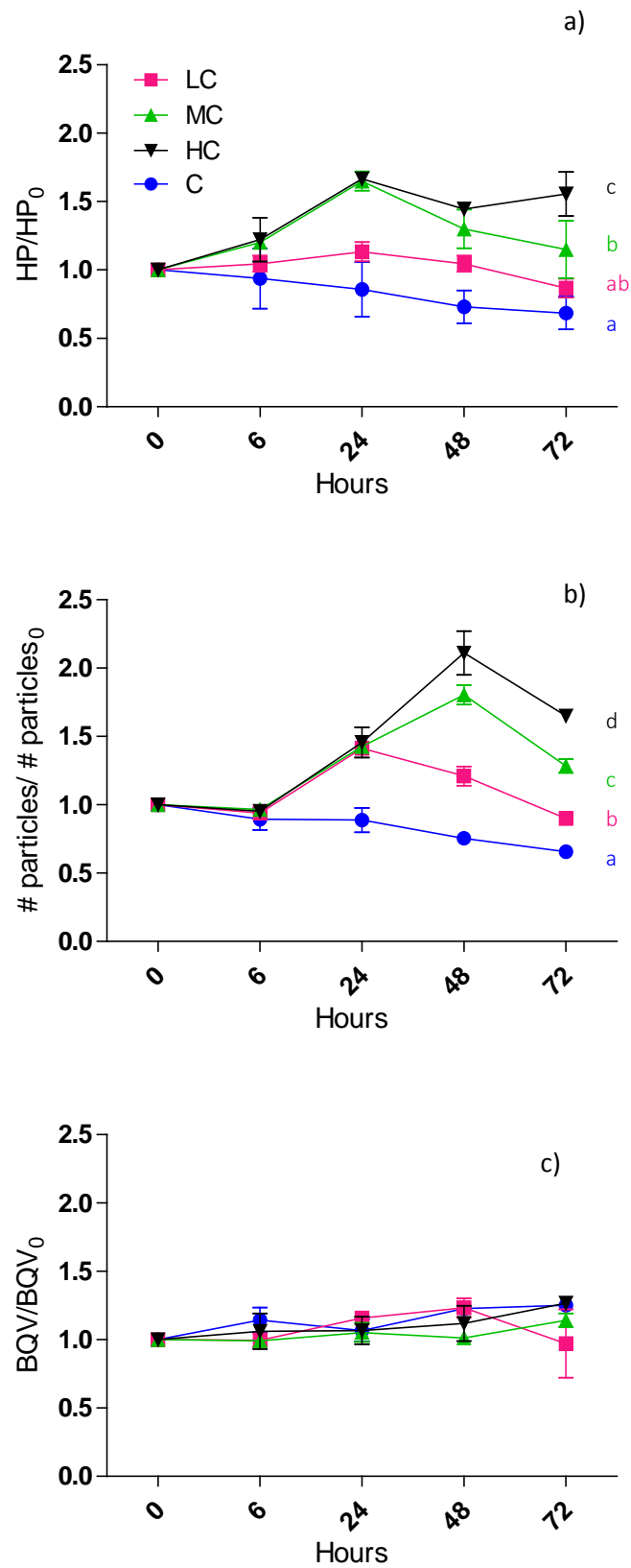


Fig. 3: Effect of acetate spike on a) hydrogen peroxide constant removal rate (k), b) number of particles with size distribution of 1 to 30 μm , and c) bactiquant value (BQV). Data normalized (C/C_0) presented as Mean \pm SD, $n = 2$.

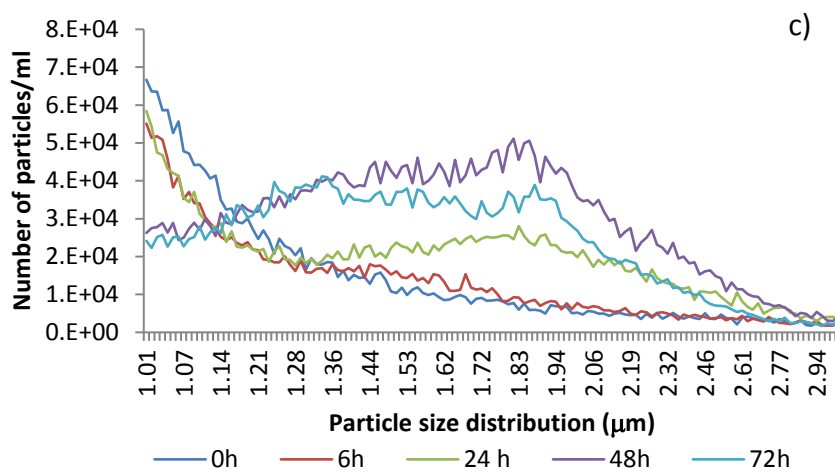
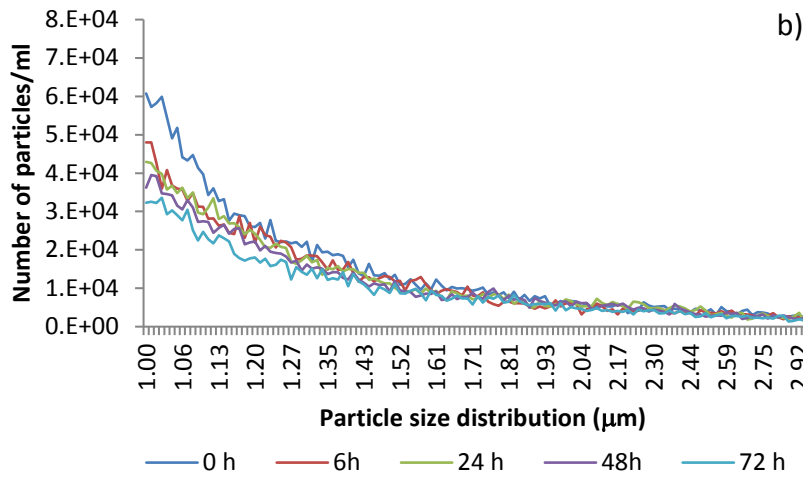
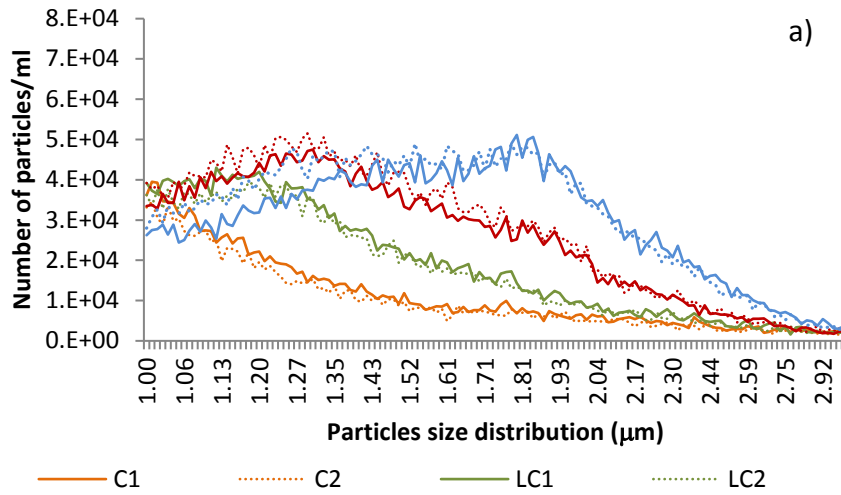


Fig. 4: Particles number and size distribution (1 to 3 μm) development accordingly to acetate concentration: a) Changes in size distribution accordingly to the different acetate concentrations after 72 h exposure, b) development of particles in one of the control beakers, and c) development of particles in one of the high acetate concentration beakers.

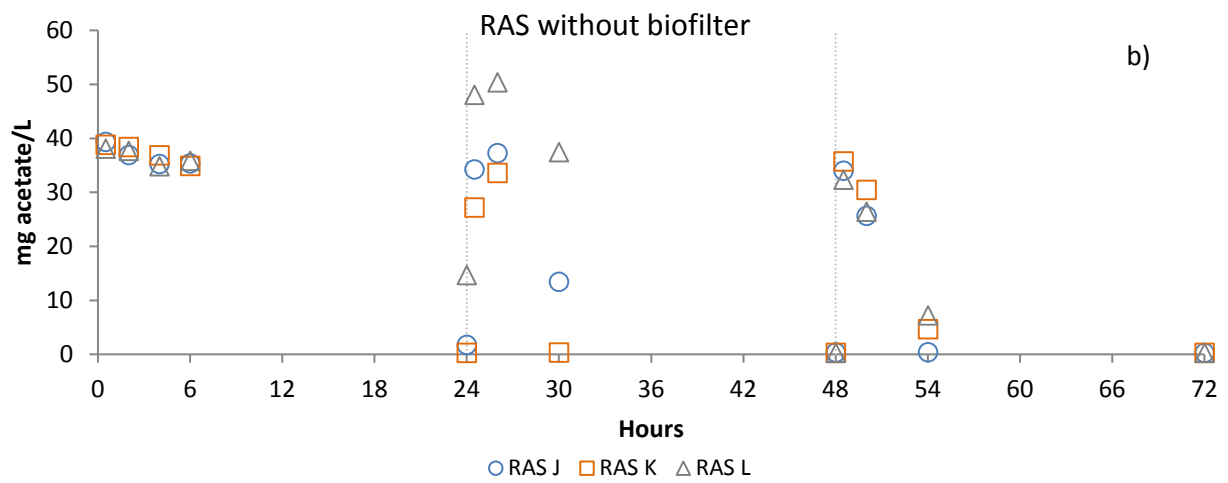
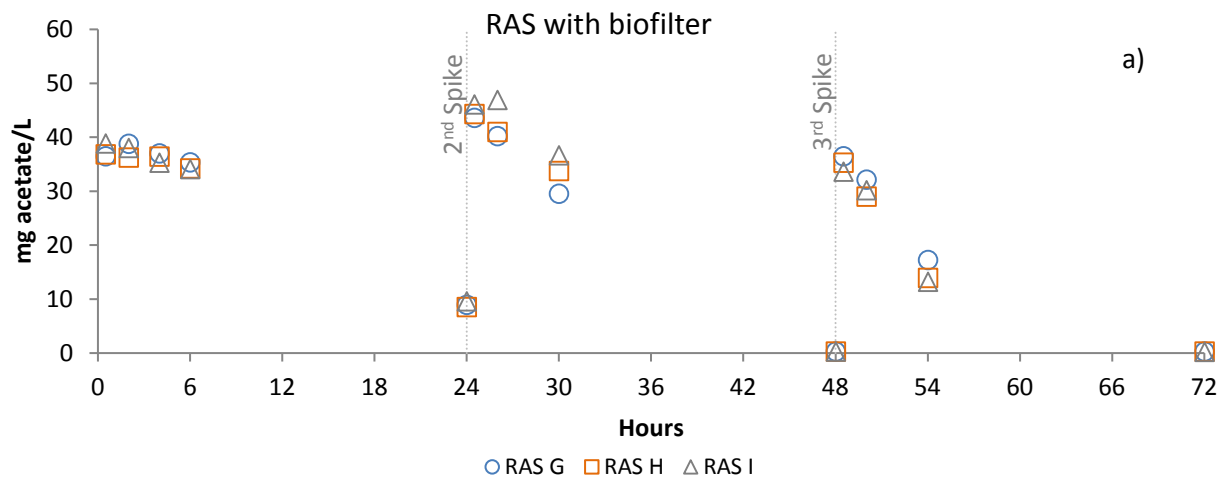


Fig 5: Measurement of acetate spike (t = 0, 24 and 48h) and degradation in: a) RAS with biofilter media and b) RAS without biofilter media.

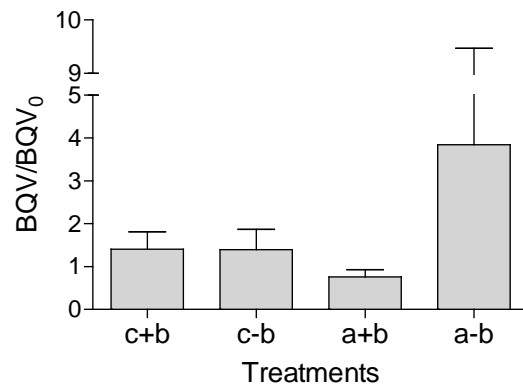


Fig. 6: Bacterial activity changes at the end of the trial (after 3 days) based on Bactiquant® method (BQV) for different treatment groups (mean ± SD, n=3): control with biofilter (c+b), control without biofilter (c-b), systems spiked with acetate with biofilter (a+b), and systems spiked with acetate with biofilter (a-b).

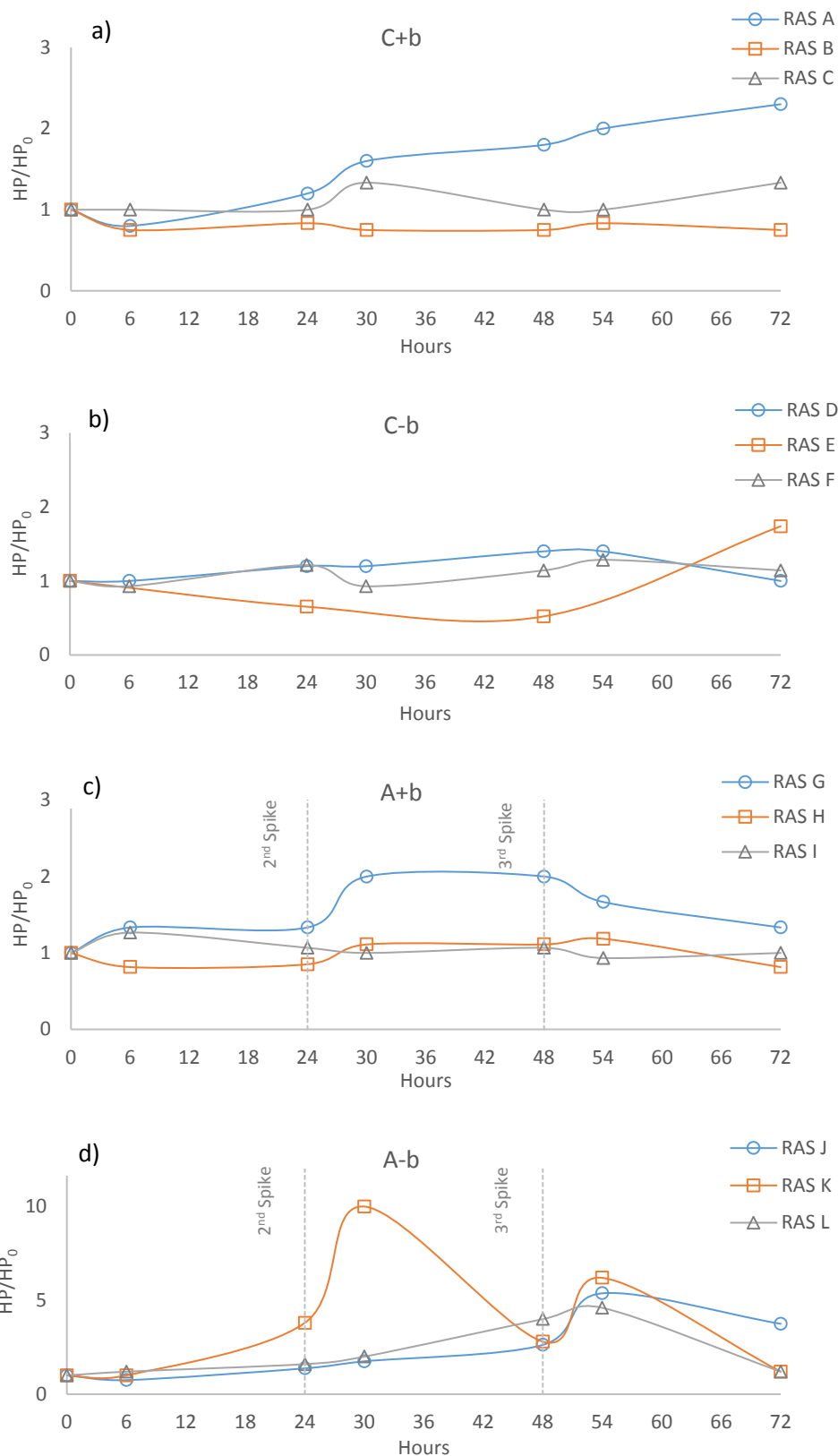


Fig. 7: Bacterial activity changes based on changes in removal rate constant k value (h^{-1}) for each RAS during 72 hours. a) Control RAS with biofilter, b) Control RAS without biofilter, c) RAS with biofilter spiked with acetate at $t = 0, 24, 48$ h, and d) RAS without biofilter spiked with acetate at $t = 0, 24, 48$ h. NOTE different scale on y-axis.

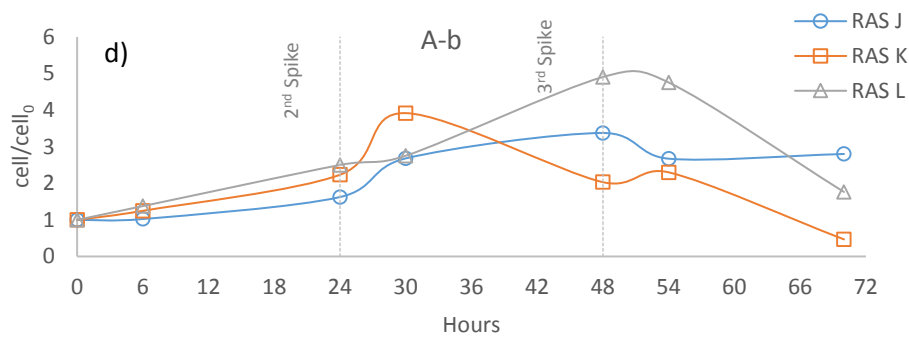
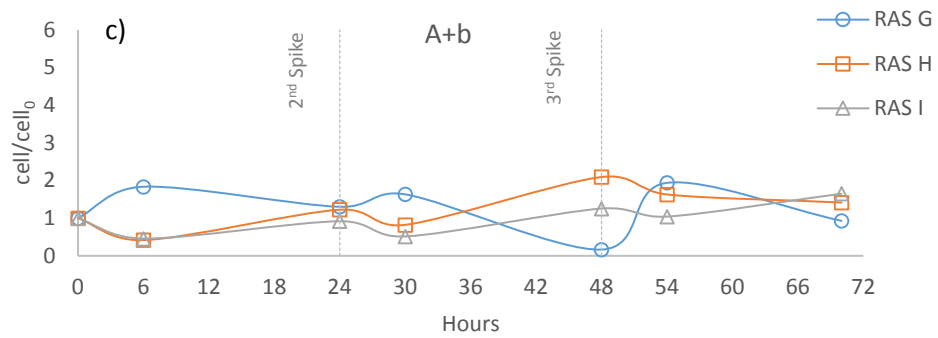
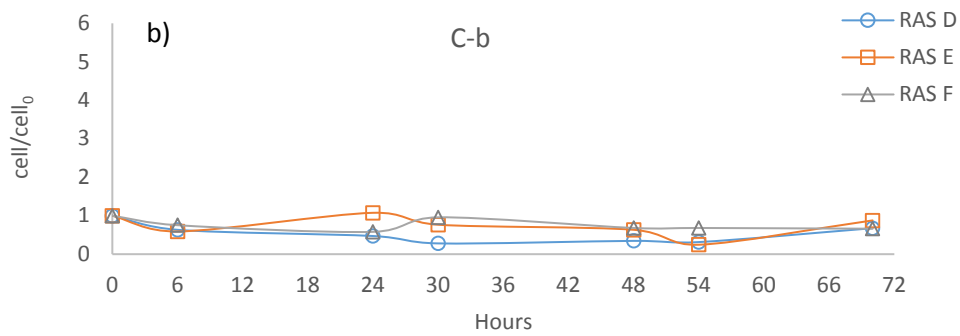
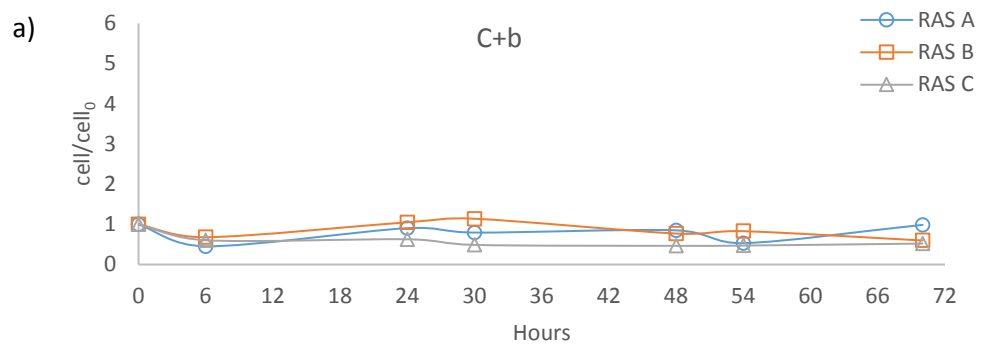


Fig. 8: Bacterial abundance changes based on flow cytometry measurements (cells/ml) for each RAS during 72 hours. a) Control RAS with biofilter, b) Control RAS without biofilter, c) RAS with biofilter spiked with acetate at t = 0, 24, 48 h, and d) RAS without biofilter spiked with acetate at t = 0, 24, 48 h.

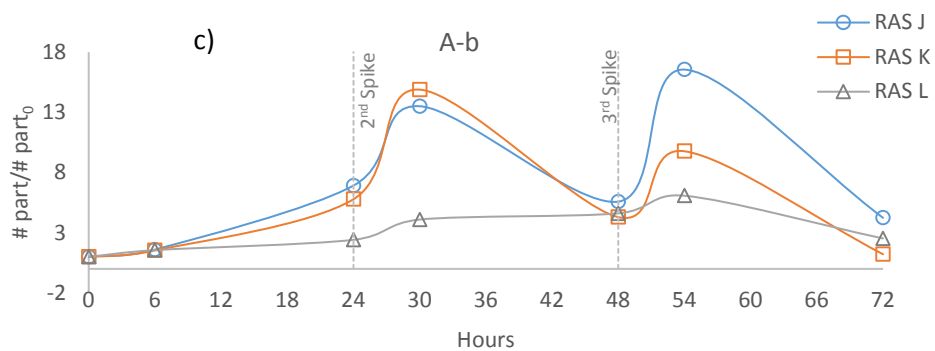
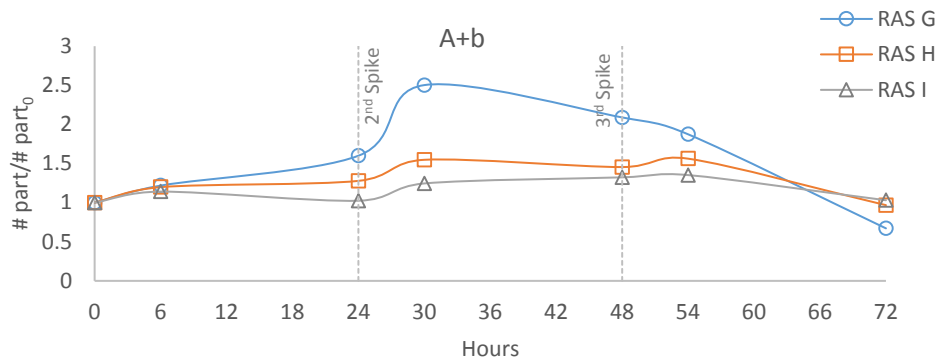
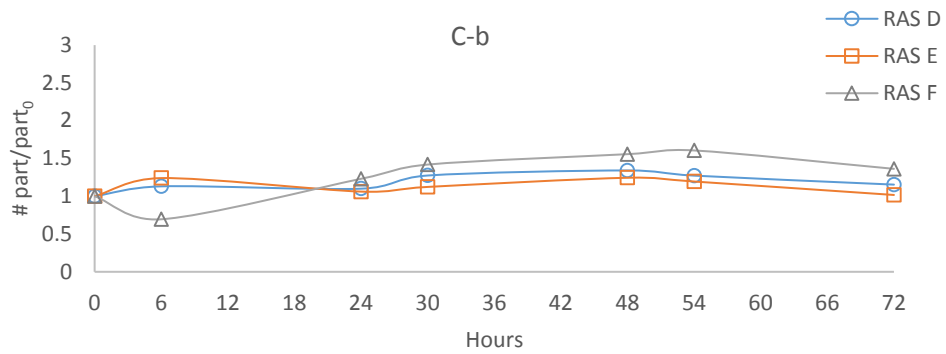
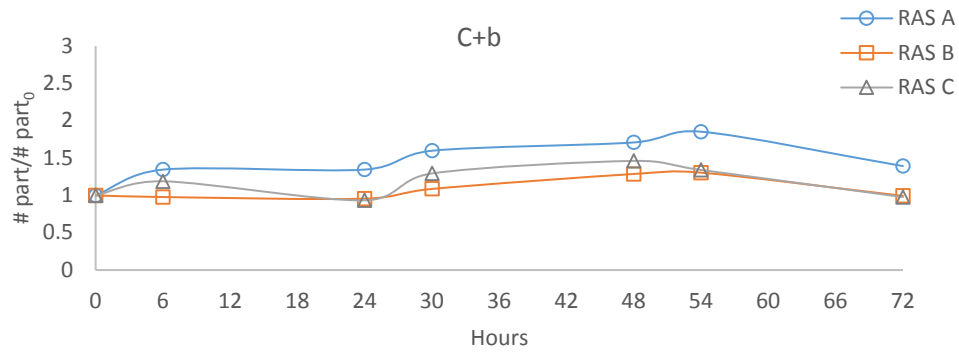


Fig 9: Changes in particle number (# part/ml) during 72 hours for: a) Control RAS with biofilter, b) Control RAS without biofilter, c) RAS with biofilter spiked with acetate at t = 0, 24, 48 h, and d) RAS without biofilter spiked with acetate at t = 0, 24, 48 h. NOTE different scale on y-axis.

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552 **Table 1: Acetate concentration used for simulating increase in organic load**

Parameters	Units	Daily feeding ^c	LC	MC	HC
Acetate	mg/L	-	10	20	40
COD	mg O ₂ /L	6 ^a	9.5	19	38
Organic carbon	mg C/L	2 ^b	4	7	14
Feed	g	125	199	399	798
Feed increase		0	× 1.6	× 3.2	× 6.4

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^a Calculated accordingly to Dalsgaard and Pedersen (2011): 81 mg O₂/ g feed.

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^b Calculated accordingly to Ebeling *et al.* (2016): 0.375 g carbon/g oxygen

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^c Daily and constant feeding for the twelve RAS.

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Table 2: Microbial and physicochemical water quality parameters and analytical methods applied.

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical Method	Reference
Bacterial Activity	BactiQuant Value	BQV	Unfiltered. Processed immediately	BactiQuant® (Mycometer, Denmark)	Manufacturers protocol
Bacterial Activity	ATP assay				
Bacteria cell number	Cell number	cell/μl	Unfiltered. Fixed with glutaric aldehyde (1% final concentration). Freezed immediately with liquid nitrogen gas and conserved at -20°C. Processed 6 months later.	Stained with Sybr Green II and counted with Flow Cytometer (Becton Dickinson FACscan)	(Marie <i>et al.</i> , 2005; Wold <i>et al.</i> , 2014)
HP degradation	HP	HP mg/L	Unfiltered. Processed immediately	Colorimetry	Arvin and Pedersen, 2015
Particle numbers	PSD		Filtered to 45 μm. Counted immediately	Multi CoulterCounter	N/A
Temperature, pH, Dissolved Oxygen	Temp., pH, O ₂	°C , pH units, mg/L	N/A	Hach HQ40d instruments, Hach Lange, Germany	N/A
Total ammonia nitrogen	TAN	mg/L	Filtered 0.22 μm. Conserved at 4°C.	Vandundersøgelse. Bestemmelse af ammonium-nitrogen.	af DS 224
Nitrite	NO ₂ -N	mg/L	Filtered 0.22 μm. Conserved at 4°C.	Vandundersøgelse. Bestemmelse af summen af nitrit- og nitratnitrogen.	af DS 223
Dissolved chemical oxygen demand	COD _{DISS}	mg O ₂ /L	Filtered 0.22 μm. Conserved at 4°C.	LCK 914, Hach Lange, Germany	N/A

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Table 3: Results from the beaker trial for the microbial water quality parameters

Parameter	Unit	Time	C	LC	MC	HC
Bactiquant	BQV/ml	0 h	6.8 - 7 × 10 ⁴	7.1 × 10 ⁴	7.1 - 7.6 × 10 ⁴	6.9 × 10 ⁴
		72 h	8.3 - 9 × 10 ⁴	5.7 - 8.1 × 10 ⁴	7.9 - 9.0 × 10 ⁴	8.6 - 8.9 × 10 ⁴
HP	h ⁻¹	0 h	0.75 ± 0.13	0.69 ± 0.04	0.60 ± 0.00	0.54 ± 0.00
		72 h	0.51 ± 0.04	0.60 ± 0.08	0.69 ± 0.13	0.84 ± 0.08
Micro particles	# particles/ml	0 h	1.9 – 2.0 × 10 ⁶	2 × 10 ⁶	1.9 – 2.0 × 10 ⁶	1.9 – 2.0 × 10 ⁶
		72 h	1.2 - 1.4 × 10 ⁶	1.8 - 1.9 × 10 ⁶	2.4 - 2.6 × 10 ⁶	3.2 - 3.3 × 10 ⁶

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